

## CHARACTERISATION OF VIRAL HAEMORRHAGIC SEPTICAEMIA (VHS) VIRUS ISOLATES

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### Abstract

A new strain of Viral Haemorrhagic Septicaemia virus (VHSV), described as VHSV strain Wi, was isolated from rainbow trout in Germany. Isolates of this virus strain did not react in the indirect immunofluorescence assay (IIFA) with the commercially available anti-VHSV monoclonal antibodies (MAbs). We could identify this new VHSV isolates by IIFA only with a MAb, named ID8, developed in our laboratory. This new VHSV strain Wi could also be identified with other authorised diagnostic methods such as virus neutralisation tests or enzyme linked immunosorbent assay, with reverse transcriptase-dependent polymerase chain reaction, immunoblot, and electron microscopy EM immunoelectron microscopy. No morphological differences between rhabdovirus strains were detectable with EM. The new VHSV variant was detected for the first time in 1992.

### Introduction

The fish farms in Germany are supervised on occurrences of Viral Haemorrhagic Septicaemia (VHS) and Infectious Haematopoietic Necrosis (IHN) on a regular basis. In Germany, the "Fischseuchenverordnung" (anonymous, 1994), which refers to the European Union (EU) legislation (anonymous, 1991, 1992, 1993) is the basis for control of fish diseases. From more than 3000 pools of organ samples of fish which were examined virologically by the regional diagnostic laboratories in 1996 a total of 133 were positive for VHS virus (VHSV) or IHN virus (IHNV) (Fichtner et al., 1996; Bergmann et al., 1997). Selected virus isolates were submitted to the National Reference Laboratory for confirmation of the diagnosis or for further characterisation using a panel of additional methods (Table 1). During this work, a VHSV strain with a different reaction pattern to commercially available monoclonal antibodies (MAbs) was detected. A further characterisation of this strain was the aim of this work.

### Material and methods

*Cell cultures:* The following cell lines were used for virus cultivation: Epithelioma

papulosum cyprini (EPC), fathead minnow (FHM), blue gill fry (BF-2) and rainbow trout gonad (RTG-2) (Fryer and Lannan, 1994). Cells were grown in 75 cm<sup>2</sup> cell culture flasks (Falcon, United Kingdom) and in 24 or 96 well cell culture plates (Greiner, Germany) at 20°C.

*Viruses:* The virus isolates D43/93, D123/93, D35/94, D72/94, D12/95, D17/95, D08/96, D53/96 and D01/97 were obtained from regional diagnostic laboratories of different German federal states (Bundeslaenders). The following reference strains were used: VHSV 123/3, belonging to serogroup F1, and VHSV strain Fi13 (Enzmann and Bruchhof 1989). For propagation of virus cell cultures were incubated at 15°C.

*Indirect immunofluorescence assay (IIFA):* The IIFA was performed according to the protocol described in European Community Commission Decision 92/532/EEC (anonymous, 1992). Freshly prepared cell suspensions were infected with virus and seeded into 96-well cell culture trays. Commercially available MAbs against VHSV and IHNV (BIO X, Belgium) and anti-VHSV MAb ID8, produced in our laboratory, were diluted 1:20. The fluorescein isothiocyanate

**Table 1.** Number of isolates examined by National Reference Laboratory for Fish Virus Diseases from 1994 until 1996

	1994	1995	1996
Isolates in total	15	22	35
Isolates positive for VHSV	10	7	21
Isolates with different reaction pattern against MAb (strain Wi)	1	2	8
Isolates positive for IHNV	3	4	2
Isolates positive for IPNV	0	2	6

**Table 2.** Identification of VHSV isolates by several diagnostic methods

Methods	Reference strains Fil3, 123/3	Isolates <sup>1)</sup> D43/93, D123/93, D35/94	Isolates <sup>2)</sup> D72/94, D12/95, D17/95, D53/96, D53/96, D01/97
IIFT with anti-VHSV MAb Bio X	+	+	Ø
IIFT with anti-VHSV MAb ID8	+	+	+
VNT with rabbit anti-VHSV serum	+	+	+
VNT with rabbit anti-IHNV serum	Ø	Ø	Ø
Antigen ELISA	+	+	+
RT-PCR	+	+	+
IB with rabbit anti-VHSV Fil3serum	+	+	+
IEM with anti-VHSV MAb ID8	+	+	+

- 1) Isolates of normal wild type virus with conventional reaction patterns against MABs  
 2) Isolates of variant VHSV strain Wi with different reaction patterns against MABs

**Table 3.** Mortality of rainbow trout after infection with VHSV isolates and the reference VHSV strain Fil3

VHSV strains	Number of rainbow trout	Time of incubation (d)	Mortality (%)	Virus reisolation
D53/96 <sup>1)</sup>	39	6	97	VHSV
D35/94 <sup>2)-</sup>	20	8	94	VHSV
FI13	32	6	100	VHSV

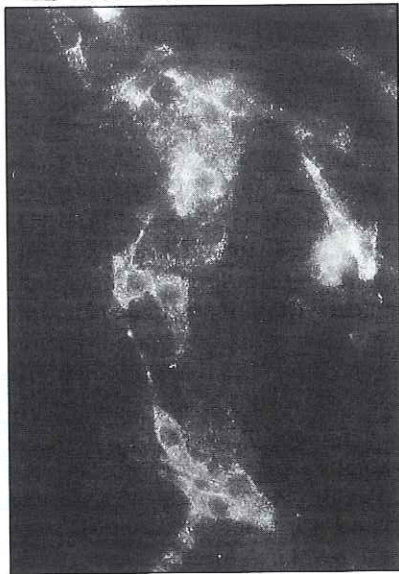
- 1) Isolate of variant VHSV strain Wi with different reaction patterns against MAB  
 2) Isolate of normal wild type virus with conventional reaction patterns against MAB

labelled goat anti-mouse immunoglobulin (DAKO, Denmark) was diluted 1:100.

*Virus neutralisation test (VNT)*: The test was performed according to the Commission Decision 92/532/EEC (anonymous, 1992). The antiserum F38 against VHSV from rabbit and the anti-IHNV rabbit serum F33 were diluted 1:50. Both sera were obtained from Dr. N. J. Olesen (EU-Reference Laboratory

for Fish Diseases, Aarhus, Denmark). 24-well cell culture plates were used for this test.

*Enzyme-linked immunosorbent assay ELISA*: The ELISA kit from TEST-Line (Czech Republic) was used for the detection of VHS V, according to the protocol recommended by the supplier. The principle of the test is described by Rodak *et al.* (1988).

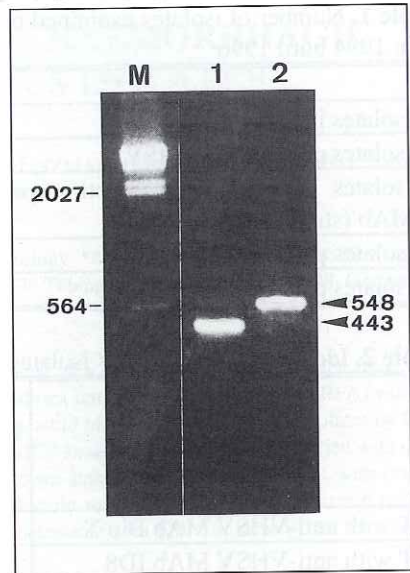


**Figure 1** Reactivity of VHSV strain Wi with Mab ID8 by IIFA(RTG-2 cells, VHSV isolate D72/94, 440x

*Reverse transcriptase-dependent polymerase chain reaction (RT-PCR) and DNA-sequencing:*

Virus growth, RNA extraction and cDNA synthesis by RT-PCR was described recently (Bruchhof *et al.*, 1995). Primers were designed from published sequences (Schütze *et al.*, 1995; Schütze *et al.*, 1996) and were synthesised on an Applied Biosystems synthesiser. Nucleotide sequences were determined by automated sequence analysis with the Taq Di-Deoxy Terminator cycle sequencing kit (Applied Biosystems) as recommended by the manufacturer. PCR amplification involved 25 cycles of 45 s at 94 °C, 1 min at 55 °C, and 1.5 min at 72 °C. Primers used for diagnosis of VHSV were as follows: sense primer: 5'-TCCCGC-TATCAGTCACCAG-3'; anti-sense primer: 5'-TGTGATCATGGTCTGGTG-3', resulting in a PCR product with 443 bp.

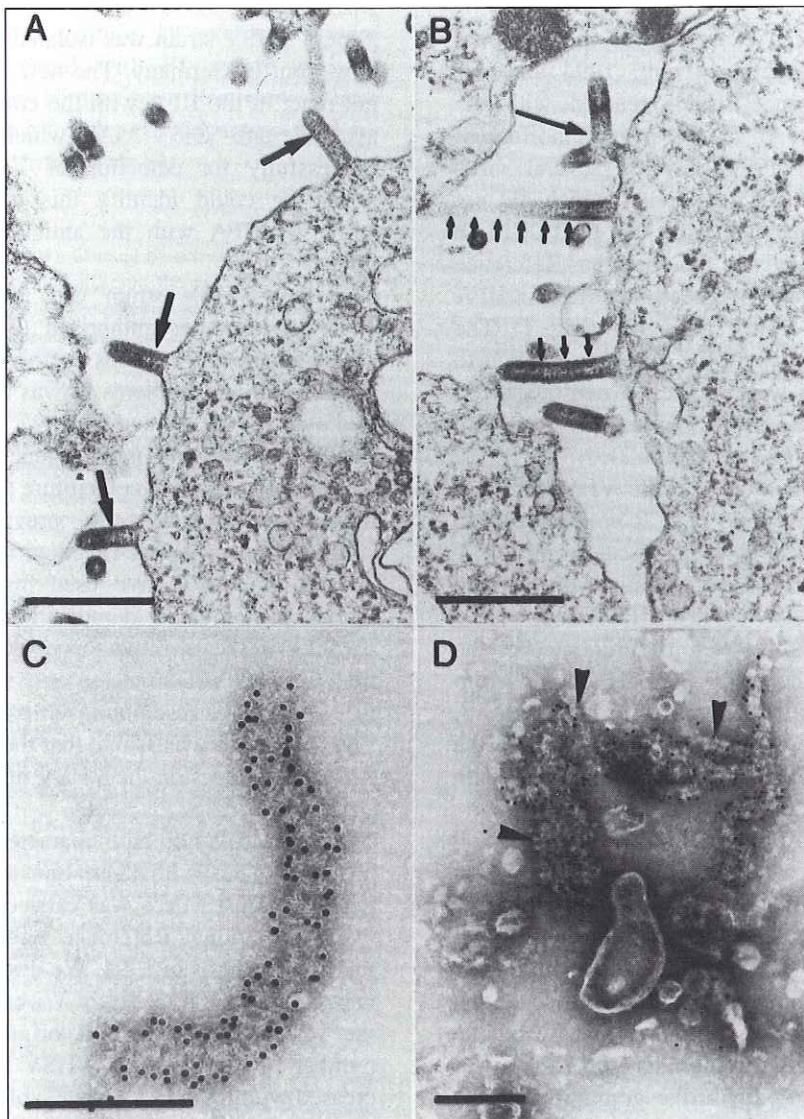
*Immunoblot (IB):* After concentration by ultracentrifugation the viral preparations were separated by polyacrylamide gel elec-



**Figure 2** Analysis of PCR products on agarose gels. M: size markers Lambda *Hind* III, lane 1: VHSV-specific PCR product with 443 bp; lane 2: IHNV-specific PCR product with 548bp for comparison purposes.

trophoresis (PAGE), transferred onto nitrocellulose sheets and labelled with anti-VHSV or anti-IHNV serum (F38, F33) and goat-anti-rabbitperoxidase-conjugate.

*Electron microscopy (EM):* Embedding of infected cell cultures for conventional electron microscopy was performed as previously described by Granzow *et al.* (1997). For immunoelectron microscopy (IEM) cell monolayer which showed a cytopathogenic effect was scraped off the plate and centrifuged at low speed. The resulting pellet was resuspended in 0.2 ml buffer and adsorbed to formvar coated, carbon stabilised and glow discharge pretreated 400 mesh copper grids. After incubation with the Mab ID8 and after several washings the primary antibody was decorated by gold-tagged anti-species antibody (GAM<sub>10</sub>, 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, Phillips, The Netherlands).



**Figure 3** Ultrathin sections of cells infected with D53/96 revealed typical rhabdoviruses (arrows) at the surface of the cell after budding (A) as well as filamentous (short arrows) and branched (long arrows) virions (B). Immunogold electron microscopy of cell sediments after infection with VHSV Fi13 ( C) and D53/96 (D) revealed distinct and characteristic labelling of rhabdovirus nucleocapsid structures with MAb ID8 (arrowheads). Bars= 500nm (A&B) and 250nm (C&D)

*Animal experiments:* 39, 20 and 32, respectively, rainbow trout with a weight of about 6g were infected by bath with  $10^4$  TCID<sub>50</sub>/ml of the VHSV isolates D53/96, D35/94 as

well as with the reference VHSV strain Fil3 for 1 h, thereafter, they were kept at 10°C for 28 d.

### Results

VHSV isolates examined in the National Reference Laboratory until 1994 and our VHSV reference strains reacted with the anti-VHSV MAb BIO X in immunofluorescence. From 1994 onwards several virus isolates from VHS outbreaks which did not show any reaction with the BIO X-MAb have been sent to the German Reference Laboratory and have been identified in IIFA only by anti-VHSV MAb ID8 (Fig. 1). The VHSV isolates with the different reaction to BIO X-MAb were, however, neutralised by the rabbit anti-VHSV serum F38 and were detectable by ELISA (Table 2).

Closer examination of these VHSV isolates by RT-PCR resulted in PCR products of 443 bp (Table 2, Fig. 2) as is typical for VHSV.

Using IB, G and N proteins were stained with rabbit sera against IHNV and VHSV (Table 2). Viruses were differentiated by the reaction of the VHSV and IHNV antiserum only against the homologous N protein. A strong serological cross-reaction against the protein G of both viruses was observed with both antisera.

Analysis of ultrathin sections of infected cell cultures revealed all stages of rhabdovirus replication as described for fish pathogenic rhabdoviruses (Fig. 3). No differences between rhabdovirus strains were morphologically detectable. Preparations of infected cell cultures for immuno-gold marker technique with MAb ID8 revealed distinct reactions of the antibody with fibrillar granular aggregations (Fig. 3). No other structures of the cells or intact virions were labelled by this antibody. Control experiments revealed no un-specific background labelling.

94-100 % of rainbow trout died in a period of 3 weeks after infection with the reference virus Fil3, the normal wild type virus isolate D35194, and the isolate D53/96 of the new VHSV variant. Infected animals showed behaviour, external and internal signs typical for VHS and VHSV could be reisolated from dead fish (Table 3).

### Discussion

A new VHSV strain was isolated from rainbow trout in Germany. The new variant did not react in the IIFA with the commercially available anti-VHSV MAb, which was used successfully for detection of VHSV until now. We could identify this new VHSV strain by IIFA with the anti-VHSV MAb ID8.

This new VHSV strain was identified as VHSV with other authorised (anonymous, 1992) diagnostic methods such as VNT and ELISA without problems. It was also identified by RT-PCR, IB and EM.

EM studies on the morphogenesis of this variant virus strain in cell culture revealed no differences compared to previously described fish pathogenic rhabdoviruses (Granzow *et al.*, 1997). We used immuno-gold marker technique for demonstration of virus particles in the EM and for virus identification. The MAb ID8 reacted only with aggregated structures resembling virus nucleocapsids. Therefore we assume that the MAb ID8 detected epitopes on the VHSV nucleocapsid structure.

The first isolation and characterisation of VHSV isolates with these unusual reaction pattern against MAbs was carried out at the German National Reference Laboratory on the Isle of Riems in 1994. We designated the new strain as VHSV strain Wi according to the village from where it originated. The number of these variant VHSV isolates increased within the last years (Table 2).

Retrograde examinations demonstrated that the first isolation of the VHSV strain Wi was performed in 1992. The virus was found as a contaminant together with the first IHNV strain in Germany (Enzmann *et al.*, 1992).

Differences in virulence were not observed between the new VHSV strain Wi in comparison to the normal wild type virus as well as of VHSV laboratory strain by experimental animal infection trials.

The VHSV isolate D12/95 of the strain Wi was examined using molecular biological

methods. In the deduced amino acid sequence of G protein 19 exchanges were found compared with F1 -strain Fil3 of VHSV. For comparison, Rindsholm virus from Denmark differs by 14 amino acid replacements from strain Fl.

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