Vol. 72: 201–211, 2006 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published October 27

Viral haemorrhagic septicaemia (VHS) outbreaks in Finnish rainbow trout farms

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ABSTRACT: In Finland, viral haemorrhagic septicaemia virus (VHSV) was diagnosed for the first time in 2000 from 4 rainbow trout farms in brackish water. Since then the infection has spread and, by the end of 2004, VHSV had been isolated from 24 farms in 3 separate locations: 2 in the Baltic Sea and 1 in the Gulf of Finland. The pathogenicity of 3 of these isolates from 2 separate locations was analysed in infection experiments with rainbow trout fry. The cumulative mortalities induced by waterborne and intraperitoneal challenge were approximately 40 and 90%, respectively. Pair-wise comparisons of the G and NV gene regions of Finnish VHSV isolates collected between 2000 and 2004 revealed that all isolates were closely related, with 99.3 to 100% nucleotide identity, which suggests the same origin of infection. Phylogenetic analysis revealed that they were closely related to the old freshwater isolates from rainbow trout in Denmark and to one old marine isolate from cod in the Baltic Sea, and that they were located close to the presumed ancestral source. As the Finnish isolates induce lower mortality than freshwater VHSV isolates in infection experiments, they could represent an intermediate stage of marine isolates evolving towards pathogenicity in rainbow trout.

KEY WORDS: Viral haemorrhagic septicaemia virus · VHSV · Rainbow trout · Epidemiology

INTRODUCTION

Viral haemorrhagic septicaemia (VHS) is a severe viral fish disease that causes significant losses in farmed rainbow trout *Oncorhynchus mykiss* around Europe. VHS virus (VHSV) is also widespread in the marine environment. The first detection of VHSV in wild fish in the marine environment was in cod *Gadus morhua* in 1979 (Jensen & et al. 1979, Jørgensen & Olesen 1987), and the number of VHSV isolates from wild marine fish species is increasing steadily (Skall et al. 2005a).

VHSV has been isolated from free-living marine fish species in the northern hemisphere near Europe, Japan and North America. Amongst the VHS positive fish species discovered in the North Sea, Baltic Sea and North Atlantic are haddock *Melanogrammus aeglefinus*, sprat *Sprattus sprattus*, dab *Limanda limanda*, Norway pout *Trisopterus esmarkii*, plaice *Pleuronectes platessa*, rockling *Rhinonemus cimbrius*, whiting *Merlangius merlangus*, blue whiting *Micromesistius poutassou* and lesser argentine *Argentina sphyraena* (Smail 1995, Mortensen et al. 1999, Smail 2000, King et al. 2001, Brudeseth & Evensen 2002, Skall et al. 2005b).

Many of the North American VHSV strains have been isolated in association with high mortality in different wild fish species, including Pacific herring *Clupea pallasi* (Meyers et al. 1994, 1999). These strains

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also induce mortality in experimental infections (Kocan et al. 1997). In contrast, the European marine VHSV isolates originating from wild fish have exhibited no or very low pathogenicity in rainbow trout in immersion experiments (Dixon et al. 1997, Skall et al. 2004).

The role of these virus strains in maintaining infection in the marine environment remains unclear. Several VHSV strains isolated from fish farms have a high level of genetic similarity with some wild marine fish VHSV isolates (Einer-Jensen et al. 2004, Snow et al. 2004). The close genetic similarity of the pathogenic and non-pathogenic VHSV strains indicates that only small differences in the virus genome may be involved in the determination of VHSV virulence for rainbow trout (Betts & Stone 2000). Since RNA viruses are known to be highly adaptable and to have high mutation rates, VHSV in wild marine fish could pose a permanent threat to rainbow trout farming, especially in the marine environment.

VHSV is an enveloped negative-strand RNA virus belonging to the genus *Novirhabdovirus* of the family *Rhabdoviridae* (Walker et al. 2000). The VHSV genome is a non-segmented, single-stranded RNA molecule with a length of approximately 11 200 nucleotides. The genome consists of 6 genes in the order 3′-N-P-M-G-NV-L-5′, encoding 5 structural proteins: nucleocapsid- (N), phospho- (P), matrix- (M), glyco- (G) and RNA polymerase (L) protein and 1 non-structural (NV) protein (Schütze et al. 1999).

VHSV isolates have been shown to cluster into 4 different genotypes, which seem to correlate with the geographical regions of isolations (Snow et al. 1999, 2004, Einer-Jensen et al. 2004). Genotype I includes a wide range of virus strains originating from freshwater rainbow trout farms in continental Europe that belong to Subgroup Ia (Benmansour et al. 1997, Stone et al. 1997, Snow et al. 1999, 2004, Thiery et al. 2002, Einer-Jensen et al. 2004). In addition, 69 isolates from marine species in the Baltic Sea/Skagerrak/Kattegat, 1 isolate from the English Channel and 2 isolates from rainbow trout farmed in the Kattegat appear to have the same ancestral source (Snow et al. 1999, 2004, Einer-Jensen et al. 2004), and belong to Subgroup Ib within Genotype I. Genotype II includes some marine isolates from the Eastern Gotland Basin and the vicinity of the island of Bornholm (Snow et al. 1999, 2004, Einer-Jensen et al. 2004). Genotype III consists of isolates originating from outbreaks of VHS in turbot farms in the British Isles (Ross et al. 1994, Einer-Jensen et al. 2004), isolates from a variety of marine species caught in Scottish waters and the Skagerrak (Snow et al. 1999, 2004, Einer-Jensen et al. 2004), and 1 isolate from an eel *Anguilla anguilla* captured in the River Loire estuary in France (Thiery et al. 2002, Einer-Jensen et al. 2004). Genotype IV includes isolates from wild marine fish in the northwest Pacific (Benmansour et al. 1997, Stone et al. 1997, Snow et al. 1999, 2004, Nishizawa et al. 2002) and isolates from Japanese flounder *Paralichthys olivaceus* (Takano et al. 2000) and Pacific sand eel *Ammodytes personatus* caught in Japan (Watanabe et al. 2002).

In Finland, VHS was diagnosed and VHSV isolated for the first time in spring 2000 from 4 rainbow trout farms in brackish water. Thereafter the infection spread, and at the end of 2004 VHSV had been isolated from 24 farms in 3 separate locations in Finland. The origin of the infections has not been determined. In this study, experimental infection trials in rainbow trout were performed to test the pathogenicity of Finnish VHSV strains. Furthermore, the epidemiology of the disease was studied by comparing the nucleotide sequences of the G and NV genes of the VHSV isolates collected from infected farms between 2000 and 2004 with each other and with available sequences in GenBank.

MATERIALS AND METHODS

History of Finnish VHS outbreaks. The first Finnish VHS outbreaks on rainbow trout farms occurred almost simultaneously in late April and May 2000 in 2 separate areas. The farms were situated 330 km apart, one in the Åland islands and the other on the coast of the Gulf of Finland. Farms in these regions produce rainbow trout in net cages for human consumption. The first outbreak occurred in the Åland islands in the southwestern Finnish archipelago. The second outbreak was diagnosed in 2 separate farms in Pyhtää on the southeastern coast of Finland, 1 mo after the first outbreak (Fig. 1). Restriction zones 40 km in diameter were immediately established around these farms. Subsequent outbreaks occurred in Pyhtää, with 1 new infected farm in late 2000 and 1 in 2001. In Åland, the infection occurred at 4 new farms in 2001. Consequently, the restriction zone was expanded to cover the whole province of Åland. The number of new infected farms in Åland reached a peak in 2002, when 12 new positive farms were confirmed. In 2003 and 2004, only 1 previously VHS-positive and 1 new farm were diagnosed as VHS-positive. In spring 2003, the infection had spread to a third location on the west coast of Finland (Uusikaupunki, Pyhämaa) due to a confirmed contact with Åland. All infected farms were located in brackish water. The Finnish VHSV strains analysed in this study consist of virus isolates from these farms (see Table 2).

Histological examination. Tissue samples of liver, anterior and posterior kidney, heart and brain were

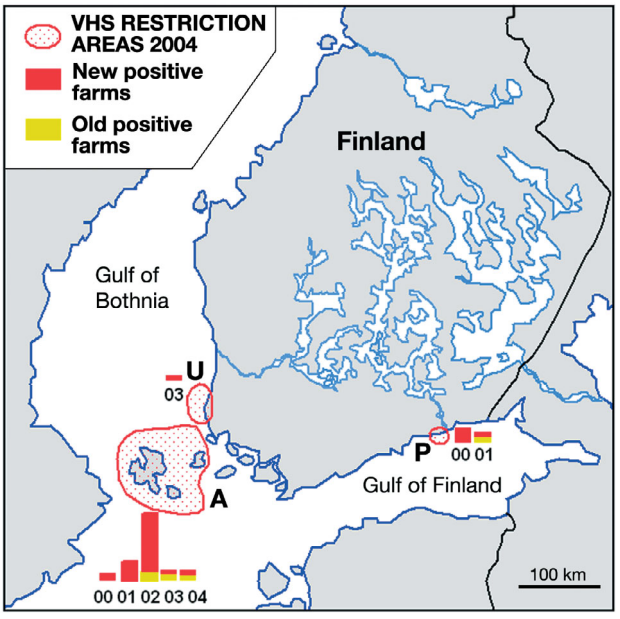

Fig. 1. Positive VHSV farms in Finland between 2000 and 2004. Total number of infected farms in each year: Åland islands (A), 2000: 1, 2001: 4, 2002: 14, 2003: 2, 2004: 2; Pyhtää (P), 2000: 3, 2001: 2, 2002–04: 0; Uusikaupunki (U) 2000–02: 0, 2003: 1, 2004: 0

fixed in 10% formalin, embedded in paraffin, sectioned at 4 µm and stained with haematoxylin and eosin (H&E).

Virus isolation and identification. Pooled heart, anterior kidney, spleen and brain tissue from 10 fish were homogenized in 9 volumes of cell culture medium (Eagle's MEM, Gibco plus 8 to 10% foetal bovine serum; pH 7.2 to 7.4) containing penicillin and streptomycin. Homogenates were centrifuged (15 min, 4000 × *g*, 4°C) and inoculated onto subconfluent monolayer cell cultures of bluegill fry fibroblast (BF-2) (Wolf & Quimby 1966) and *Epithelioma papulosum cyprini* (EPC) (Fijan et al. 1983) in 24-well dishes (Nunc A/S) in final dilution ratios of 1:100 and 1:1000. Inoculated cultures were incubated in a CO_2 incubator at 16°C and inspected regularly with a microscope for the occurrence of cytopathic effect (CPE) at 40× magnification. When total CPE was evident, the cell culture medium was collected.

Staining of cells for immunofluorescence: The indirect fluorescent antibody technique (IFAT) was used for VHSV identification. Cover glass (diameter 13 mm, Menzel-Glaser) cultures of EPC cells grown in 24-well dishes were inoculated at dilutions of 10^{-1} and 10^{-2}. Uninfected cells served as negative controls. The infected cultures were fixed with acetone on Days 1 and 2 post-infection and the IFAT technique performed as described previously (Lorenzen et al. 1988). Briefly, the monoclonal antibody (MAb) 1P5B11 (DFVF, Århus) against the nucleocapsid (N) protein of the VHSV-F1 strain was diluted in phosphate-buffered saline and applied as the first antibody on the monolayer. After 30 min incubation at 37°C, diluted fluorescein isothiocyanate (FITC)-labelled rabbit anti-mouse immunoglobulin was added as the second antibody (DAKO A/S) and incubated for 30 min at 37°C. The monolayer was washed and examined under an epifluorescence microscope. VHSV was used as a positive control and infectious hematopoietic necrosis virus (IHNV), infectous pancreatic necrosis virus (IPNV) and uninfected cells as negative controls.

ELISA: Culture media (50 µl) from cells cultures showing evidence of CPE were analysed with a commercial ELISA kit according to the manufacturer's instructions (Test-Line) to confirm the presence of VHSV.

Neutralisation pattern: The neutralisation pattern was characterised by a 50% plaque neutralisation test (50%PNT) against a panel of neutralising monoclonal antibodies as previously described (Olesen et al. 1993).

Infection trials. Infection trials in rainbow trout were performed to test the pathogenicity of the Finnish VHSV strains. The rainbow trout were infected intraperitoneally (i.p.) or by bath infection.

Fish: Rainbow trout fingerlings were 1.6 g (SD = 0.6 g) in size and were purchased from a Danish commercial fish farm, officially registered free of IPN and VHS, 1 wk before infection.

Tanks: Tanks contained 8 l of soft freshwater (2.5 to 4.5°H) and water temperature was maintained at 9.5 to 11°C. The water used was groundwater of drinking quality (not chlorinated), and new water was added continuously with no recirculation. The water was aerated and tanks were closed with a transparent lid to avoid aerosols and to prevent fish from jumping out.

Virus isolates: A list of virus isolates used and their origin is presented in Table 1. All isolates used in infec-

Table 1. VHSV isolates tested in infection trials. All isolates originated from outbreaks in farmed rainbow trout *Oncorhynchus mykiss*

Isolate	Origin	Water condition	Year of isolation	Source
FiA01a.00	Baltic Sea, Finland: Åland area	Brackish water	2000	This study
FiP02a.00	Baltic Sea, Finland: Pyhtää area	Brackish water	2000	This study
FiP02b.00	Baltic Sea, Finland: Pyhtää area	Brackish water	2000	This study
DK-3592B positive control	Denmark	Fresh water	1986	Lorenzen et al. (1993)

tion trials were of low passage numbers (max. 5 passages) and were propagated and titrated on BF-2 cells, according to standard procedures (Lorenzen et al. 1988).

Positive and negative controls: The highly pathogenic VHSV strain DK-3592B (Lorenzen et al. 1993) isolated from a rainbow trout farm was used as a positive control by immersion only. As a negative control, Eagle's MEM with Tris-buffer and 10% new-born calf serum (dilution medium) was used by immersion and i.p. injection.

Trials: Each group was tested in duplicate with 35 to 40 fish in each tank. Dead fish were removed daily from each tank. The number of dead fish and clinical symptoms of VHS in fingerlings was recorded daily. At the end of the trial, surviving fish were euthanized with an overdose of benzocaine and counted. The sum of surviving fish and fish that died during the trial equalled the number of fish in each tank. Trials ended after 21 d.

Immersion trial: To obtain a concentration of 10^5 $TCID_{50}$ ml^{-1} water, virus isolates were added to a vial containing 10 ml dilution medium according to their respective titres. Water supply was stopped while virus dilutions were added to each tank, for an exposure time of 2 h, after which the continuous water flow was resumed.

Intraperitoneal (IP) injection trials: Virus isolates were mixed in vials with a 10 ml dilution medium to obtain a dose of 10^5 $TCID_{50}$ per fish when injected into a volume of 50 µl. Fish were anaesthetised by bathing them in benzocaine solution, injected intraperitoneally with a new needle and syringe for each isolate and transferred to tanks supplied with running fresh water.

Virus isolation: Fish that died and survivors at the end of the trials were sampled for virus isolation. Negative and positive control groups were also sampled. Fish were frozen at –25 or –80°C for later examination. Fish from the trial groups were examined in pools of 3 to 8 (immersion groups, mortalities and survivors), 3 to 9 (i.p. injection, mortalities) or 1 to 5 fish (i.p. injection, survivors). The positive control groups were examined for mortalities in pools of up to 10 fish. Fish from the negative control groups were examined individually for mortalities and in pools of 5 fish for survivors.

Tissues (heart, kidney and spleen) were ground in a mortar and pestle with sterile sand, diluted to a ratio of 1:10 in a dilution medium, treated with gentamycin overnight and inoculated on BF-2 cells, as described previously (Mortensen et al. 1999). Virus was identified as VHSV by ELISA as described by Skall et al. (2004).

Statistical analysis: Cumulative mortalities at the end of the trial within and between different groups in each experiment were compared by chi-squared analysis (Dunn 1977). If $p < 0.05$, the difference was regarded as significant.

RNA extraction. Finnish VHSV isolates from the period 2000 to 2004 (Table 2) were included in this study. The cell culture medium (200 µl) collected from virus isolation experiments (usually 1 to 3 passage numbers) were used for RNA extraction with RNA affinity spin columns (RNeasy Total RNA kit; Qiagen), according to the manufacturer's instructions. Purified RNA samples were redissolved in 30 µl sterile water containing RNase inhibitor, and stored until use at –70°C.

Table 2. Finnish VHSV-positive farms from 2000 to 2004. Isolate names coded as follows: Fi = Finland, A = Åland islands, P = Pyhtää, U = Uusikaupunki; the first 2 numbers refer to the infected fish farm in the restriction area; a_b_c = separate isolations from the same farm; the last 2 numbers refer to the year of isolation; e.g. isolates FiP01a_b_c.00 were collected and isolated from Pyhtää Farm 01 on 3 different occasions in 2000. GenBank accession numbers provided in parentheses

Year	Location	Isolate
2000	Åland Islands	FiA01a_b.00 (AM086354)
	Pyhtää	FiP01a_b_c.00 (AM086355)
		FiP02a_b.00 (AM086356)
		FiP03.00 (AM086357)
2001	Åland Islands	FiA02a.01 (AM086358)
		FiA02b.01 (AM086359)
		FiA03a_b.01 (AM086360)
		FiA04.01 (AM086361)
		FiA05.01 (AM086362)
	Pyhtää	FiP04.01 (AM086363)
		FiP03.01 (AM086364)
2002	Åland Islands	FiA03.02 (AM086365)
		FiA04.02 (AM086366)
		FiA06.02 (AM086367)
		FiA07.02 (AM086368)
		FiA08.02 (AM086369)
		FiA09.02 (AM086370)
		FiA10.02 (AM086371)
		FiA11.02 (AM086372)
		FiA12.02 (AM086373)
		FiA13.02 (AM086374)
		FiA14.02 (AM086375)
		FiA15.02 (AM086376)
		FiA16.02 (AM086377)
		FiA17.02 (AM086378)
2003	Åland Islands	FiA03.03 (AM086379)
		FiA18.03 (AM086380)
	Uusikaupunki	FiU01.03 (AM086381)
2004	Åland Islands	FiA03.04 (AM086382)
		FiA19.04 (AM086383)

RT-PCR amplification and sequencing. Sequences of the entire G and NV genes and the region between them (G-NV region) were examined in this study. Standard RT-PCR methods were used to amplify overlapping sequences from the gene regions studied (Sambrook & Russel 2001) (Thermal cycler PTC-1000, MJ Research.). Briefly, cDNA was prepared from purified RNA using *MuLV* reverse transcriptase (Applied Biosystems) and Random Hexamers (Applied Biosystems) as primers. Following reverse transcription, cDNA was used as a template for PCR amplifications with *Dynazyme II* polymerase (Finnzymes). Several primers (Table 3) designed to amplify overlapping regions of the G and NV gene sequences were used in this study. They were designed on the basis of the published genomic sequences (accession numbers in Table 4) and sequence data generated in this study. At least 2 independent amplification events were performed for each gene region, in order to eliminate errors introduced by the polymerase. After PCR, amplified sequences were purified using Microspin S-400 HR Columns (Amersham Biosciences). The quality and quantity of amplicons were evaluated in 1% agarose gel electrophoresis, after which the products were sequenced directly. Sequencing reactions were performed using a ABI PRISM 3100-Avant Genetic Analyzer using the PRISM Big Dye v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequences of both strands of PCR products were determined using the same primers as in PCR. Sequences were analysed using Sequencing Analysis Software version 5.1 (Applied Biosystems).

Table 3. Primers used for PCR amplification and sequence analysis. The complete published genome (GenBank accession no. Z93414) was used as a basis to calculate positions for primers. The relative position is given with respect to the open reading frame of each gene

Primer[a]	Position (nt)	Relative position (nt)	Primer (5′ → 3′)
G1+	2600	501–518, M gene	CGGGCAGGCGAAGGACTA
G2+	2794	1–21, G gene	ATGGAATGGAATACTTTTTTC
G3+	3371	578–600, G gene	CAACCTCGCCCTGTCAAACTCAT
G4+	3616	823–840, G gene	GGGGGCGCGAGGAAACTG
G5+	3816	1023–1042, G gene	TGGACCCGGCAAGGCACACT
G1–	3073	260–282, G gene	CGGAGACGCTGGTGACTGATA
G2–	3490	678–697, G gene	TGTGATCATGGGTCCTGGTG
G3–	3996	1180–1203, G gene	GTCCCCAAATATCATCCCATCGTA
G4–	4317	1504–1524, G gene	TCAGACCGTCTGACTTCTGGA
NV+	4008	1215–1238, G gene	CCCGGACATCGAGAAGTATCAGAG
NV–	5000	96–113, L gene	GCCAAGCGCCCGAAGAGC

[a]Forward primers marked +, reverse primers marked –

Table 4. Other VHSV isolates used for phylogenetic analysis

Isolate code	Year of isolation	Origin	Host species	Genotype	GenBank accession no.
DK-F1	1962	Denmark	Rainbow trout	I	AY356633
NO-A163-68	1968	Norway (probably imported from Denmark)	Rainbow trout	Id	AY546621
FR-07-71	1971	France	Rainbow trout	Ia	AY546616
DK-Hededam	1972	Denmark	Rainbow trout	I	Z93412
DK-M.rhabdo	1979	Denmark	Cod	Ib	AY356632
DK-3592B	1986	Denmark	Rainbow trout	Ia	X66134
UK-96-43	1996	English Channel	Herring	Ib	AF143862
DK-200070-4	2000	Denmark	Rainbow trout	Ia	AY546612
SE-SVA-1033	2000	Kattegat	Rainbow trout	Ib	AY546623
DK-1p52	1996	Baltic Sea	Sprat	II	AY546576
DK-1p53	1996	Baltic Sea	Herring	II	AY546577
DK-4p168	1997	Skagerrak	Herring	III	AY546582
FR-L59X	1987	France	Eel	III	AY546618
US-Makah	1988	Washington, USA	Coho salmon	IV	U28747
JP-KRRV9822	2000	Japan	Japanese flounder	IV	AB179621

Computer analysis. To determine the similarity of Finnish isolates with other published VHSV isolates, basic local alignment search tool (BLAST) similarity searches were performed against GenBank. A few representative VHSV isolates from each genotype were selected (Table 4) to further analyse the genetic relationships. Multiple sequence alignments of nucleotide sequences were performed with the MegAlign program using the Clustal Method (DNASTAR) and the Clustal W program. Phylogenetic trees were constructed from G (see Fig. 3) and G-NV (see Fig. 4) gene alignments. Analysis was performed using the neighbour-joining DNA distance method with the Kimura-2-parameter in Mega version 3.0 (Kumar et al. 2004). Reliability of trees was determined by 1000 data set bootstrap resampling within the MEGA program.

RESULTS

Pathological findings and virus identification

In most of the Finnish outbreaks, the rainbow trout infected were 1 to 2 yr old. According to the farmers, the mortality rate was between 3 and 10%. However, in a few outbreaks, mortality reached almost 50%. Typical VHS lesions were observed in the post-mortem examination. The fish were anaemic and had exophthalmia and small haemorrhages in the eye orbits and skin. The peritoneum, swim bladder and visceral adipose tissue were full of petechial haemorrhages. Some of the fish had multiple petechiae in the dorsal musculature, heart and gills. The spleen was clearly enlarged and kidney slightly swollen and hyperaemic. In some fish the liver was mottled with hyperaemic areas. In histological sections, severe multifocal liver necroses and prominent necroses of the tubular cells of the kidney were seen.

The isolated viruses were identified as VHSV by indirect immunofluorescence and ELISA. Serotyping by 50%PNT demonstrated that the original isolates were neutralised at high titres against all monoclonal antibodies in the panel, and were thus grouped into Neutralisation Pattern 1 (results not shown).

Infection trials

Infected fish showed signs typical of VHS, and VHSV was isolated from dead fish in addition to most of the survivors (examined in pools of 1 to 10 fish) in tanks with fish infected by immersion and i.p.

In the immersion trial, the Finnish isolates produced a cumulative mortality of 38 to 39%. The negative control fish had a cumulative mortality of 3% and the positive control fish 98% (Fig. 2).

Chi-squared analysis revealed no significant difference in cumulative mortality between duplicate tanks.

Chi-squared analysis did not show statistical significant difference among the Finnish isolates, whereas there was a significant difference between the tested isolates and the positive and negative controls. In tanks with fish infected i.p., a cumulative mortality of >81% was detected, and average mortality was >89%. There was a statistically significant difference between duplicate tanks of Pyhtää isolate FiP02b. The negative control had a cumulative mortality of 4% and the positive control 100%.

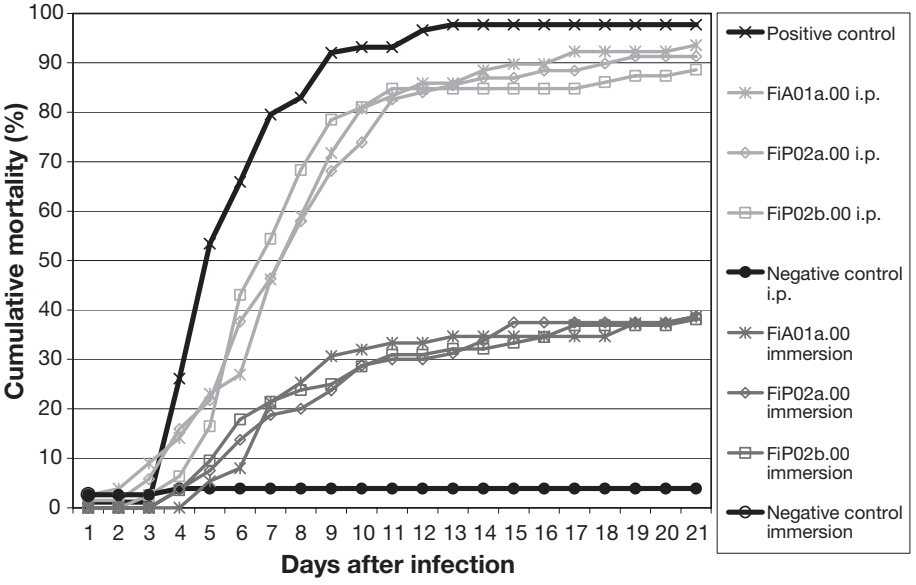

Fig. 2. Cumulative mortality induced by 3 Finnish VHS strains isolated in 2000 (FiA01a00, FiP0200a and FiP0200b) studied by immersion and i.p. injection. Pathogenic VHSV strain DK-3592B (Lorenzen et al. 1993) was used as a positive control by immersion only. Growth medium was used by immersion and i.p. injection as negative control (data points and line for negative immersion are concealed behind those for negative control i.p.). Virus dose of i.p. infections was 10^5 $TCID_{50}$ per fish in 50 µl volume. In immersion experiments, virus titre was 10^5 $TCID_{50}$ ml^{-1} water for an immersion period of 2 h. Length of the trial was 21 d

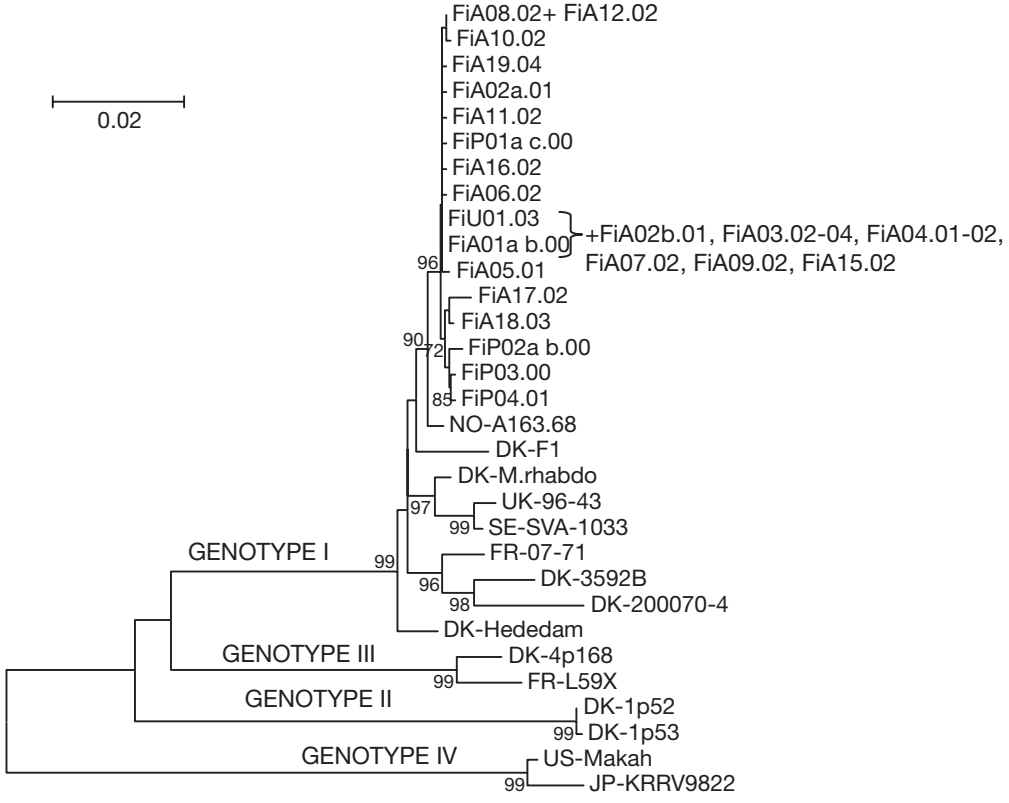

Fig. 3. Phylogenetic tree of relationships of the VHSV G gene (1524 bp) from Finnish farm isolates and a few representative isolates from all known major genotypes. Bootstrap values <70% not shown. Scale bar = expected number of substitutions per site

Genetic analysis

Sequences of the G (1524 nucleotides) and NV (369 nucleotides) genes and the region between these genes (74 nucleotides) were determined for all 34 Finnish VHS strains isolated between 2000 and 2004 from fish farms producing rainbow trout. Pair-wise comparisons of sequenced regions revealed that all isolates were closely related, with 99.3 to 100% nucleotide identity (data not shown).

Genetic relationships of the G genes among Finnish isolates with some representative isolates from the other known genotypes (Genotypes I to IV; Snow et al. 1999, 2004, Einer-Jensen et al. 2004) are illustrated in Fig. 3. All Finnish isolates were grouped together into Genotype I with a bootstrap value of 96%. They formed a subgroup (Id) with a strain isolated from rainbow trout in Norway in 1968 (NO-A163.68, supposedly imported from Denmark), as previously described by Einer-Jensen et al. (2004). When the Finnish sequences were compared with gene sequences retrieved from GenBank, the shortest genetic distances observed between the Finnish (2000 to 2004) and other isolates were with the old freshwater isolate NO-A163.68 (identity 99.1 to 99.5% in the G gene), DK-Hededam (identity 98.3 to 98.7% in the G-NV gene region) and marine isolate DK-M.rhabdo (identity 98.5 to 99.0% in the G-NV gene region) isolated in 1968, 1972 and 1979 respectively. Identity of the G gene of Finnish isolates was 97.8 to 98.4% with the first VHSV strain (DK-F1) isolated from rainbow trout in 1962, and 97.2 to 97.5% with the DK-3592B isolate used as a positive control.

The genetic relationships of all Finnish isolates are illustrated in Fig. 4, which was created from the alignments of the G-NV gene regions (total 1967 bp). From the Åland region, 26 isolates were collected between 2000 and 2004. They shared 99.6 to 99.9% nucleotide identity, except for 2 isolates (FiA17.02 and FiA18.03) that differed by 0.3 to 0.7% (6 to 14 bases) from each other and from all other Finnish isolates. Five isolates (FiA01a.00, FiA01b.00, FiA04.01, FiA04.02 and FiA09.02) from Åland were found to be identical in the G-NV gene region. The isolates from Pyhtää (n = 8) were clustered into 2 different groups. The first contained 3 identical isolates from the same fish farm (FiP01a.00, FiP01b.00 and FiP01c.00). They were closer in identity to the Åland isolates (99.7 to 99.9% identity) than to other isolates from Pyhtää. The second group from Pyhtää consisted of 5 isolates (FiP02a.00, FiP02b.00, FiP03.00, FiP04.01 and FiP03.01), which shared a 99.8 to 100% identity with each other. They differed both from the first group from Pyhtää and from isolates from Åland by 0.4 to 0.6% (8 to 11 bases). The

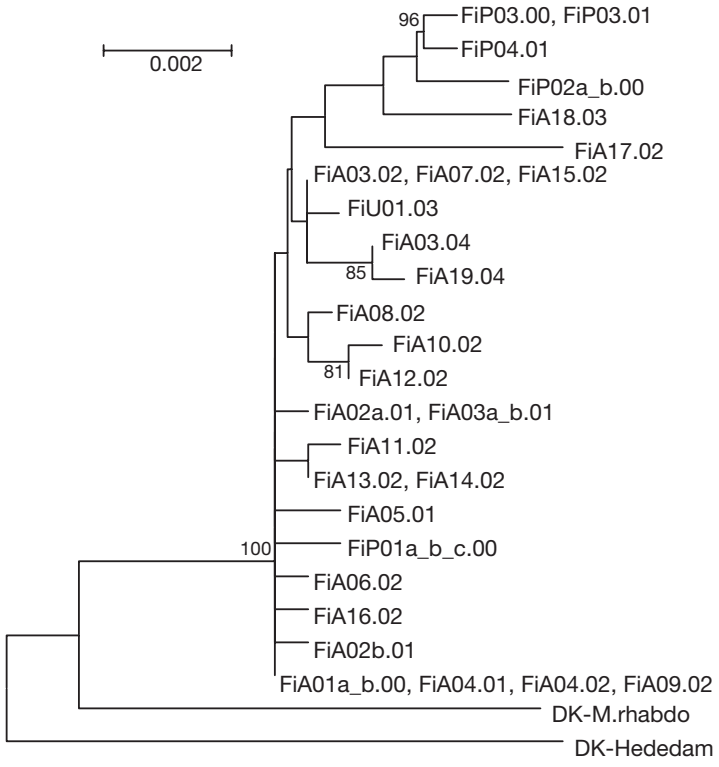

Fig. 4. Phylogenetic tree of relationships of all Finnish isolates. Tree generated from a sequence alignment of G and NV genes and the region between these genes (1967 bp). DK-M.rhabdo and DK-Hededam isolates were used as outgroups. Bootstrap values <70% not shown. Scale bar = expected number of substitutions per site

isolate FiAU01.03, collected in 2003 from the western coast of Finland (Uusikaupunki), shared a 99.7 to 99.9% identity with isolates from Åland.

VHSV was isolated several times in some fish farms. We sequenced 3 different isolates from farm P01 in Pyhtää (FiP01a.00, FiP01b.00 and FiP01c.00). The samples collected at intervals of 1 wk shared 100% homology in the gene regions studied. In Åland, isolates FiA01a.00 and FiA01b.00 (collected at an interval of 2 wk) and isolates FiA03a.01 and FiA03b.01 (collected at an interval of 1 wk) were identical within the farm. In contrast, the 2 Åland isolates FiA02a.01 and FiA02b.01 (collected at an interval of 1 wk) differed from each other by 0.1% (2 bases) in the G-NV gene region. On 2 fish farms, isolates collected from the same farm in consecutive years were identical (FiP03.00 and FiP03.01 from Pyhtää, and FiA04.01 and FiA04.02 from Åland). From one fish farm in Åland (FiA03), VHSV was isolated in 4 consecutive years. The first year isolate (FiA03.01) has 1 transition in the G gene and 1 in the NV gene when compared with subsequent isolates. The coding regions of G and NV genes in isolates FiA03.02, FiA03.03 and FiA03.04 are all identical. The only change detected within these isolates was the accumulation of 1 transition per year in the intergenic region between the G and NV genes.

In this study, the NV gene was found to be slightly more conserved than the G gene within the Finnish isolates: isolates had 99.3 to 100% identity in the G gene and 99.5 to 100% identity in the NV gene. The G gene was found to be completely identical within 12 isolates out of 34, and the NV gene within 26 out of 34. At the amino acid level, isolates had a 99.2 to 100% identity in the G protein and 98.3 to 100% identity in the NV protein. Similar phylogenetic trees were obtained using G and NV regions. The intergenic region (74 bp) showed more variation than the coding regions.

DISCUSSION

We studied the pathogenicity and molecular epidemiology of VHSV infection in Finnish rainbow trout farms, using isolates collected from 2000 to 2004. Altogether, 34 VHSV isolates were collected and their G and NV gene regions were analysed. Both gene regions were shown to be very similar among isolates, which strongly suggests the same origin of infection. In experimental infection trials, the Finnish VHSV strains induced a mortality of approximately 40% when infected by immersion, which was lower than the mortality in infection trials using VHSV isolates from recent outbreaks in freshwater rainbow trout farms (Skall et al. 2004). In contrast, VHSV strains isolated from wild populations of marine fish induced no or less than 5% mortality in rainbow trout when infected by immersion (Skall et al. 2004). Finnish isolates could represent an intermediate stage of marine strain evolving towards pathogenicity in rainbow trout. When infected by i.p. injection, the Finnish isolates were highly pathogenic.

Because of limited sequence data in GenBank, the same VHSV strains could not be used in phylogenetic analysis of different gene regions. Based on analysis of the G gene, the Finnish isolates grouped together in Genotype I, Subgroup Id, with a Norwegian isolate from 1968 supposedly imported from Denmark (Einer-Jensen et al. 2004). Genotype I also included isolates from wild fish from the Baltic Sea (Subgroup Ib), as well as other farmed rainbow trout isolates (Subgroup Ia). Finnish isolates were shown to be genetically more closely related to the old freshwater isolates and to the first marine isolate from Denmark than to recent isolates, and were thus demonstrated to be located close to the ancestral source within the phylogenetic trees (Einer-Jensen et al. 2004; Fig. 3). In this study, similar results were obtained when sequences covering the G and NV genes and the intergenic region were

compared with sequences in GenBank. The closest relative was found to be the marine isolate DK-M.rhabdo, which was isolated from cod in 1979 (Fig. 4). Despite a very close genetic relationship — a 98.4 to 98.9% identity within this gene region — the Finnish isolates caused 40% mortality in rainbow trout by immersion whereas the marine isolate DK-M.rhabdo did not cause any (Skall et al. 2004).

The first VHS strain DK-F1 is serologically and genetically closely related to the Finnish VHSV strains. Previous experimental infection studies of rainbow trout induced by the VHSV reference strain DK-F1 revealed moderate to high mortalities, ranging from 30 to 90% depending on fish size, inoculation dose and temperature (Jørgensen 1970, 1974). More recent studies (N. Olesen unpubl. data) revealed lower mortality induced by VHSV DK-F1 compared with positive control isolates. In one study comparing infectivity of 5 VHSV isolates in 0.5 g rainbow trout fry (duplicate tanks with 75 fish in each at 11 to 12°C), mortality was 98 to 100% for 3 VHSV isolates including the positive control DK-3592B, whereas mortality was only 48 to 50% in rainbow trout infected with DK-F1.

Finnish VHSV isolates are genetically so closely related that variable gene regions are difficult to find. We detected some variation at the beginning of the G gene (3 to 4 different bases) in 3 of the isolates, leading to a change in the 6th amino acid from leucine to phenylalanine.

This difference did not affect virus pathogenicity in experimental infection, as one of the Finnish isolates used in infection experiments bore this difference. In addition, the isolates used in infection experiments differed by 2 silent nucleotide changes in the G gene and 2 in the intergenic region between the G and N genes; no differences were detected in the NV gene. The previously reported variable region V2 of the G gene (amino acids 238 to 331), where many amino acid substitutions have been reported to accumulate (Benmansour et al. 1997), exhibited 100% amino acid homology in the Finnish material. The only variation observed in this region was 1 silent transition at Position 993 in 3 isolates and 1 silent transversion at Position 934 in 1 isolate. The non-coding region between the G and NV genes (74 bp) showed more variation among isolates than the coding regions, and was useful when examining similar virus strains. The role of the VHSV intergenic regions has not yet been studied, but our data suggest different selection pressures on coding and non-coding regions.

The NV gene was not optimal for phylogenetic analysis of VHSV, at least not when closely related strains were compared. In our data, the G gene varied more than the NV gene and appeared to be more useful for genetic analysis among closely related strains. The G gene was found to be identical in 12 out of 34 isolates, whereas the NV gene was identical in 26 out of 34 isolates. For example, the isolates FiA01a_b.00 and FiP02a_b.00 had 5 nucleotide differences in the G gene and none in the NV gene. The observed difference in variation may be due to different selection pressures on these genes or to the different size of the genes — the G gene is 4 times longer than the NV gene. The role of the NV protein in the viral infection cycle is not yet clear. The NV gene was highly conserved in the Finnish material, suggesting that it has an essential role. The NV protein of IHNV was shown to play an important role in the pathogenicity of the virus in rainbow trout (Thoulouze et al. 2004).

In this study the mutation rate of the VHSV was found to be low: isolates collected at different times/years from the same fish farms were almost all identical. If we assume a common ancestor for the Finnish isolates and the Norwegian isolate of 1968, the estimated nucleotide substitution rate is 1.3×10^{-4} to 2.6×10^{-4} site^{-1} yr^{-1}. This value is within the range of the lowest evolutionary rates estimated for other RNA viruses (Jenkins et al. 2002), and lower than previously reported for other VHSV isolates (Einer-Jensen et al. 2004). Very small differences in the G protein have been shown to affect the pathogenicity of rhabdoviruses (Dietzschold et al. 1983, Takayama-Ito et al. 2006). Similarly, amino acid differences within the G protein in both VHS- and IHN-viruses have been detected and implied to have a role in the pathogenicity of novirhabdoviruses (Kim et al. 1994, Benmansour et al. 1997, Gaudin et al. 1999, Betts & Stone 2000). As only single mutations in the G-region may determine the virulence of VHSV for rainbow trout, VHSV in wild marine fish can represent a continuous potential threat to rainbow trout cage farms located in the marine environment.

VHSV was not isolated in Finland before the first outbreaks in 2000, and mainland Finland still has the status of an approved VHS and IHN-free zone according to European Council Directive 91/67/EC (Anon. 1991). The origin of the first outbreaks is still unclear. Freshwater origin from inside Finland seems unlikely because the mainland is free of VHS, and farms supplying fingerlings were shown to be free of the virus. Therefore, a marine origin of infection is likely. The closest relatives of the Finnish VHSV strains were isolated in Denmark, the Baltic Sea near the Danish coast and Norway (most likely a Danish import) between 1962 and 1979. Temporal distance aside, the short genetic distance to the old Danish isolates would suggest the original source of the infection to be there. All isolates belong to Neutralisation Pattern I, together with almost all (97%) fresh water isolates from Continental Europe isolated before 1986. Almost all isola-

tions from VHS outbreaks in rainbow trout farms within the last 15 yr belong to Neutralisation Patterns II or III (>92%); therefore, recent introduction from other European countries seems unlikely. There is some genetic variation within a few of the Finnish isolates, which fits with the hypothesis of a marine source as the initial origin of the outbreaks. Assuming the infections originated from wild fish populations, e.g. herring, it is natural that some genetic variation should occur. The ancestral source of VHSV could still exist somewhere in the Baltic Sea. The Finnish outbreaks could also have resulted from a recent re-introduction from an unknown source. Are there some VHSV carriers in the pelagic fish populations that circulate close to Finnish fish farms? Another source of the outbreaks could be the feeding of fish with raw wild fish, as was previously conducted in Finnish fish farming. We know that both infected farms in Pyhtää used acid-treated Baltic herring and slaughter wastes in their semi-moist fish feed. On the first VHS-positive farm in Åland, only commercial fish feed was used, but the farm was close to a fishing harbour. It seems evident that human activities played a role in the rapid spread of the virus.

VHSV has been isolated from several fish species in the Baltic sea, e.g. herring *Clupea harengus*, sprat *Sprattus sprattus* and dab *Limanda limanda*. Candidates for fish species that could serve as hosts or carriers of VHSV in the brackish water regions surrounding Finland could be Baltic herring, sprat, dab, grayling *Thymallus thymallus*, whitefish *Coregonus lavaretus* and pike *Esox lucius.* Broodfish of salmon *Salmo salar*, grayling and whitefish from coastal regions of Finland have been sampled and monitored for VHS since 1995, with negative results (authors' unpubl. data). Studies on the possible presence of VHSV in trawled wild fish from Finnish brackish water regions will be conducted and will in the future provide information on the possible presence of VHS in different fish species.

We studied the Finnish VHS epidemic by infection trials using the first 3 isolates and by sequencing the G and NV genes from all VHSV isolates collected between 2000 and 2004. Our study represents the first time that NV gene sequences have been compared to this extent, and our results support the hypothesis that wild fish populations were the source of the primary infection.

Acknowledgements. This study was supported by the Ministry of Agriculture in Finland. Staff at the Departments of Virology and Pathology at Evira (former EELA) are acknowledged for participating in diagnosis of fish samples. We thank K. Savipakka at EELA and technicians at the DFVF Fish Section for technical assistance. K. Einer-Jensen is acknowledged for critical review and constructive criticism of the manuscript, and L. Nuotio for design of Fig. 1.

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Editorial responsibility: Carey Cunningham, Aberdeen, UK

Submitted: October 24, 2005; Accepted: August 18, 2006
Proofs received from author(s): October 17, 2006