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FISH RHABDOVIRUSES

VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUS (VHSV) AND PERCH RHABDOVIRUS (PRV): STUDY OF VIRAL STRAINS AND THE DISEASE EPIDEMIOLOGY IN FINLAND

Tuija Gadd

ACADEMIC DISSERTATION

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TO JOANNA AND TATU

"There is only one thing that makes a dream impossible to achieve:
the fear of failure" (Paolo Coelho)

ABSTRACT

Viral haemorrhagic septicaemia virus (VHSV) was diagnosed after clinical symptoms for the first time in 2000 from four rainbow trout *Oncorhynchus mykiss* (Walbaum) farms in Åland and Pyhtää in Finland. Phylogenetic analysis based on the full-length VHSV glycoprotein (G) and nonvirion (NV) genes of the Finnish VHSV isolates in 2000–2004 revealed that all isolates are closely related and grouped in the genotype Id, which suggests the same origin of infection. Finnish isolates were shown to be 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 located close to the presumed ancestral source. Infection with the VHSV genotype Id has spread since then, and the same genotype had been isolated from rainbow trout farms in three separate locations: Åland in the Baltic Sea, and Uusikaupunki in the Gulf of Bothnia, and Pyhtää in the Gulf of Finland. The majority of isolations have been from Åland, and since 2009 have only been from there. The VHSV genotype Id was isolated from Pyhtää only in 2000 and 2001 and from Uusikaupunki once in 2004 and 2008.

The pathogenicity of rainbow trout genotype Id isolates was analysed in infection experiments with rainbow trout fry. The cumulative mortalities induced by waterborne and intraperitoneal challenge were approximately from 13% to 40% and 66 % to 90%, respectively, depending on the size of the rainbow trout fingerlings. The Finnish brackish water VHSV genotype Id isolates induce lower mortality than freshwater VHSV isolates in infection experiments but they could represent an intermediate stage of marine isolates evolving towards pathogenicity in rainbow trout.

The occurrence of viral haemorrhagic septicaemia virus (VHSV) was examined in the main spawning stocks of wild European river lamprey, *Lampetra fluviatilis,* in the rivers of Finland from 1999 to 2008. In total, 2621 lampreys as 262 pooled samples were examined virologically during 1999–2008. VHSV was isolated from five lamprey samples from the mouth of the rivers Lestijoki and Kalajoki, which flow into the Bothnian Bay of the Baltic Sea from Finland. The full-length VHSV G gene sequence revealed that the isolates were closely related to the VHSV strains isolated earlier from herring and sprat, *Sprattus sprattus* (L.), in Gotland and were therefore assigned to VHSV genotype II. The virulence of the lamprey VHSV genotype II isolate was evaluated by an experimental infection trial in rainbow trout fry. No mortality was induced post-infection by either waterborne or intraperitoneal challenge.

To clarify the role of wild fish, especially Baltic herring, *Clupea harengus membras* (L.), in the epidemiology of VHSV in brackish waters, Baltic herring with no visible signs of disease were collected from the Archipelago Sea, the Gulf of Bothnia and from the Eastern Gulf of Finland. In total 7580 herring as 758 pooled

samples and 3 029 wild salmonid broodfish were virologically examined during 2004–2006. VHSV was isolated from 50 pooled herring samples collected from the Archipelago Sea and one pooled sample collected from the Gulf of Bothnia. Further studies based on the full-length VHSV G gene sequence revealed that the Finnish herring isolates were VHSV genotype II, closely related to the VHSV strains isolated earlier from herring and sprat in Gotland.

VHSV genotype II isolated from the lamprey and herring is thought to be independent of the VHSV Id epidemic in farmed rainbow trout in Finnish brackish waters. The most varied VHSV strains are found in seawater. This would indicate that the viruses in freshwater originate from the sea.

Two fish farms situated in the lake area of Finland have experienced elevated mortalities affecting fry of grayling, *Thymallus thymallus*, since 2002. These farms are using surface water for the production of juveniles of several fish species. Fourteen pooled samples were positive in virus isolation. Based on full-length G gene and partial RNA polymerase (L) gene sequences and the indirect fluorescent antibody technique (IFAT), the virus was classified as a perch rhabdovirus (PRV). Pairwise comparisons of the G and L gene regions of grayling isolates revealed that all the isolates were very closely related, with almost 100% nucleotide identity, which suggests the same origin of infection for the two farms. PRV isolates were closely related to the strain isolated from perch, *Perca fluviatilis* and sea trout, *Salmo trutta trutta*, caught from the Baltic Sea. The second shortest phylogenetic distances to rhabdoviruses isolated from other countries appeared to be to perch, grayling and pikeperch isolates from France and a pike rhabdovirus isolate from Denmark. This is the first time PRV has caused disease in grayling in Finland.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Raja-Halli M, Vehmas TK, Rimaila-Pärnänen E, Sainmaa S, Skall HF, Olesen NJ, Tapiovaara H (2006) Viral haemorrhagic septicaemia (VHS) outbreaks in Finnish rainbow trout farms. Diseases of Aquatic Organisms 72, 201–211. doi:10.3354/dao72201. www.int-res.com/articles/dao2006/72/d072p201.pdf

II Gadd T, Jakava-Viljanen M, Einer-Jensen K, Ariel E, Koski P, Sihvonen L (2010) Viral haemorrhagic septicaemia virus (VHSV) genotype II isolated from European river lamprey *Lampetra fluviatilis* in Finland during surveillance from 1999 to 2008. Diseases of Aquatic Organisms 88, 189–198. doi: 10.3354/dao02169.

III Gadd T, Jakava-Viljanen M, Tapiovaara H, Koski P Sihvonen L (2011) Epidemiological aspects of Viral haemorrhagic septicaemia virus (VHSV) genotype II isolated from Baltic herring (*Clupea harengus members* L.) Journal of Fish Diseases 2011:34,517–529. doi: 10.1111/j.1365-2761.2011.01264.

IV Gadd T, Viljamaa-Dirks S, Holopainen R, Koski P, Jakava-Viljanen M (2013) Characterization of perch rhabdovirus (PRV) in farmed grayling *(Thymallus thymallus)*. Diseases of Aquatic Organisms. doi:10.3354/dao02654 (in press).

The publications are referred to in the text by their Roman numerals. The original publications are reprinted with the permission of their copyright holders: Inter Research (**I, II, IV**) and Wiley (**III**).

ABBREVIATIONS

AngRV	Anguillid rhabdovirus
BF-2	Bluegill fry (cell line)
CHN	China
CHSE-214	Chinook salmon embryo (cell line)
ca.	circa (Latin), about
χ^2	chi-squared
CMC	cell-mediated cytotoxicity
CPE	cytopathic effect
DEN	Denmark
DNA	deoxyribonucleic acid
DTU	Technical University of Denmark
EELA	National Veterinary and Food Research Institute
e.g.	exempli gratia (Latin), for example
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
EPC	Epithelioma papulosum cyprini (cell line)
EURL	Community reference laboratory for fish diseases
EVA	Eel virus American
EVEX	Eel virus European X
Evira	Finnish Food Safety Authority
ex vivo	Latin: “out of the living”
FHM	Fathead minnow (cell line)
FI or Fi	Finland
FITC	fluorescein isothiocyanate
FRA	France
G	glycoprotein
HIRVV	Hirame rhabdovirus
ICTV	International Committee on Taxonomy of Viruses
i.e.	id est (Latin), that is
IFAT	indirect fluorescent antibody test
IFN	interferon
IgG	immunoglobulin G
IgM	immunoglobulin M
IHN(V)	Infectious haematopoietic necrosis (virus)
IM	intramuscular
IP	intraperitoneal

IPN(V)	Infectious pancreatic necrosis (virus)
ISG	IFN stimulated gene
ITA	Italy
kb	kilobase
kDa	kilodalton
L	large protein or polymerase
M	matrix protein
MAb	monoclonal antibody
MCP	major capsid protein
MEM	minimum essential medium
ML	maximum likelihood
MP	maximum parsimony
mRNA	messenger ribonucleic acid
N	nucleoprotein
NCC	nonspecific cytotoxic cells
NED	Netherlands
NJ	neighbour-joining
NK	natural killer
nt	nucleotide(s)
NV	nonvirion
OIE	World Organisation for Animal Health (Office International des Epizooties)
ORF	open reading frame
P	phosphoprotein
p.i.	post infection
PAb	polyclonal antibody
PCR	polymerase chain reaction
PFRV	Pike fry rhabdovirus
PG	Pike gonad (cell line)
PNT	plaque neutralisation test
PRV	Perch rhabdovirus
qPCR	quantitative polymerase chain reaction
RLFP	restriction fragment length polymorphism
RNA	ribonucleic acid
RTG-2	Rainbow trout gonad (cell line)
RT-qPCR	real-time quantitative polymerase chain reaction
RT-PCR	reversed transcriptase polymerase chain reaction
SCRV	Siniperca chuatsi rhabdovirus
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFRV	Starry flounder rhabdovirus
SHRV	Snakehead rhabdovirus

SMRV	Scopthalmus maximus rhabdovirus
SSN-1	Snakehead-fish (cell line)
SSTV	Swedish sea trout virus
STRV	Sea trout rhabdovirus
SVCV	Spring viraemia of carp virus
TCID50	50% tissue culture infective dose
VHS(V)	Viral haemorrhagic septicaemia (virus)
YUG	Yugoslavia

1 INTRODUCTION

Rhabdoviruses (family *Rhabdoviridae*) are enveloped negative-strand RNA viruses that belong to the order *Mononegavirales*. Rhabdoviruses are bullet or cone-shaped (from vertebrates and invertebrates) (Ge et al. 2010) or bacilliform (from plants) (Jackson et al. 2005). These morphological characteristics have served as the basis for primary classification purposes compared to other families (Wagner 1987). Further studies, performed for different rhabdoviruses, have demonstrated their antigenic relatedness (Shope et al. 1970; Shope et al. 1979; Shope 1982; Calisher et al. 1989). In addition, phylogenetic comparisons of available rhabdovirus genome fragments have suggested a monophyletic origin (Bourhy et al. 2005).

The *Rhabdoviridae* include pathogens of many animals and plants, including humans, livestock, fish and crops, with significant public health, veterinary and agricultural impacts. The oldest and most famous rhabdovirus is rabies virus. Over 200 rhabdoviruses have been identified, but most of them are poorly described (Dietzgen et al. 2011). Viruses classified as *Rhabdoviridae* are currently grouped into nine genera on the basis of structural properties, antigenicity and phylogenetic analyses (Dietzgen et al. 2011; ICTV 2013a). Many viruses of the genera *Vesiculovirus*, *Lyssavirus*, *Ephemerovirus and Tibrovirus* are transmitted by arthropods, whereas lyssaviruses are transmitted directly between mammals by bite and fish rhabdoviruses are waterborne (Dietzgen et al. 2011; Dietzgen & Kuzmin 2012). The remaining five rhabdovirus genera are more taxon-specific in their host preference. *Novirhabdovirus* and *Perhabdovirus* infect numerous species of fish, while *Cytorhabdovirus* and *Nucleorhabdovirus* are arthropod-borne, infecting plants, and *Sigmavirus* infect representatives of *Diptera* (Dietzgen & Kuzmin 2012).

Fish rhabdoviruses have been well documented since the 1950s (Tordo et al. 2004; Kurath & Winton 2008). Three distinct genera of fish rhabdoviruses have been identified: *Novirhabdovirus* and *Perhabdovirus*, representatives of which have all been isolated from fish hosts, and *Vesiculovirus* (ICTV 2013a). *Infectious hematopoietic necrosis virus* (IHNV), *Viral haemorrhagic septicaemia virus* (VHSV) and *Hirame rhabdovirus* (HIRVV) are widespread and economically significant novirhabdoviruses. *Perch rhabdovirus* (PRV) as the type species and both *Anguillid rhabdovirus* (AngRV) and *Sea trout rhabdovirus* (STRV) belong to the new genus *Perhabdovirus*. *Spring viraemia of carp virus* (SVCV) was the first fish virus species to be assigned to the genus *Vesiculovirus* (Carstens 2010). Infection with these viruses generally results in an acute haemorrhagic syndrome with septicaemia, ascites, petechiae, ecchymoses, necrotic ulceration and severe

bleeding of internal organs associated with mortality (Kimura et al. 1986; Johnson et al. 1999; Ahne et al. 2002; Fu 2005; Hoffmann et al. 2005).

Three rhabdoviruses have so far been found in Finland. VHSV was diagnosed for the first time in spring 2000 from four rainbow trout (*Oncorhynchus mykiss)* farms around Pyhtää, on the southern coast, and Åland. In northern Finland, STRV was isolated from farmed brown trout, *Salmo trutta* m. *lacustris*, in 1987 and named as brown trout rhabdovirus ka 903_87 (also known as lake trout rhabdovirus, LTRV or 903/87) (Koski et al. 1992). Mortalities were detected in grayling (*Thymallus thymallus*) fry caused by PRV in eastern Finland. Both STRV and PRV belong to the new genus *Perhabdovirus*. The viral strains and disease epidemiology of VHSV and PRV in Finland were investigated in this thesis research.

2 REVIEW OF THE LITERATURE

2.1 RHABDOVIRUSES OF FISH WITHIN THE FAMILY *RHABDOVIRIDAE*

Rhabdoviruses have been very successful at evolving and diversifying within the fish host (Walker & Winton 2010). Fish viruses have evolved with low temperature optima for replication, ranging from 12–25 °C, and they are inactivated at temperatures above 20–30 °C. Fish rhabdovirus diseases are not zoonoses.

The International Committee on Taxonomy of Viruses (ICTV) approved the genus *Novirhabdovirus* as the first taxonomic genus within the fish rhabdoviruses (Walker et al. 2000). All members of the *Novirhabdovirus* genus are fish pathogens. The genus was named after the presence of a unique gene called the non-virion (NV) gene, which is found between the glycoprotein (G) and polymerase (L) genes (Kurath & Leong 1985; Benmansour et al. 1997). It has four important type species: *Infectious hematopoietic necrosis virus* (IHNV), *Viral haemorrhagic septicaemia virus* (VHSV), *Hirame rhabdovirus* (HIRVV) and *Snakehead rhabdovirus* (SHRV) (Table 1). IHNV and VHSV are important pathogens of salmonids, but can also infect a very wide range of other fish species (Walker & Winton 2010). HIRVV and SHRV are pathogens of marine fish in Asia (Johnson et al. 1999; Kim et al. 2005). The transmission of fish novirhabdoviruses occurs directly through mucosal surfaces or the skin by immersion in infected water (Ahne et al. 2002).

A new genus of fish rhabdoviruses recognised since 2013 is *Perhabdovirus*. It has three species: *Perch rhabdovirus* (PRV) as the type species, *Anguillid Rhabdovirus* (AngRV) and *Sea trout rhabdovirus* (STRV). Perhabdoviruses share morphological characteristics, genome organization and sequence similarities with vesiculoviruses and with viruses in the newly proposed genus *Sprivivirus* (proposal under revision) (ICTV 2013b). The genus *Vesiculovirus* comprises an ecologically diverse but genetically similar group of mammalian and fish rhabdoviruses. It currently contains the recognised species *Spring viraemia of carp virus (SVCV)* as the type species and the tentative vesiculoviruses pike fry rhabdovirus (PFRV). However, the newly proposed genus *Sprivivirus* (ICTV 2013c) consists of both SVCV and PFRV. A growing number of fish viruses are related to viruses from the genus *Vesiculovirus*, such as *Siniperca chuatsi* rhabdovirus (SCRV) and starry flounder rhabdovirus (SFRV) (Tao et al. 2008; Talbi et al. 2011). The *Scopthalmus maximus* rhabdovirus (SMRV), originally isolated from diseased cultured turbot *Scophthalmus maximus* with lethal haemorrhagic disease in China, also has a phylogenetic relationship with the genus *Vesiculovirus*, but is genetically distinct from other rhabdoviruses (Zhang et al. 2007; Zhu et al. 2011). Transmission of fish vesiculoviruses can occur

by immersion in infected water and does not appear to involve arthropod vectors (Ahne et al. 2002).

Table 1. Rhabdoviruses isolated from fish (modified from tables in Mork et al. 2004, Hoffmann et al. 2005, Kuzmin et al. 2009, Talbi et al. 2011 and ICTV 2013b).

Genus and species	Abbreviation	Reference
Novirhabdovirus		
Viral haemorrhagic septicaemia virus (Egtved virus)	VHSV	Jensen (1963)
Brown trout rhabdovirus (VHSV)		de Kinkelin & Le Barre (1977)
Cod ulcus syndrome rhabdovirus (VHSV)		Jensen et al. (1979)
Carpione brown trout rhabdovirus (VHSV)	583	Bovo et al. (1995)
Infectious hematopoietic necrosis virus	IHNV	Amend et al. (1969)
Oregon sockeye virus (IHNV)	OSV	Wingfield et al. (1969)
Sacramento River chinook virus (IHNV)	SRCV	Wingfield & Chan (1970)
Hirame rhabdovirus	HIRVV	Kimura et al. (1986)
Snakehead rhabdovirus	SHRV	Ahne et al. (1988)
Eel virus B12	EV-B12	Castric et al (1984)
Eel virus C26	EV-C26	Castric et al (1984)
Rio Grande perch rhabdovirus		Malsberger & Lautenslager (1980)
Vesiculovirus (Sprivivirus as a new proposal)		
Spring viremia of carp virus (Rhabdovirus carpio)	SVCV	Fijan et al. (1971)
Swim bladder inflammation virus (SVCV)	SBI	Bachmann & Ahne (1973)
Pike fry rhabdovirus	PFRV	de Kinkelin & Le Barre (1973)
Grass carp rhabdovirus (PFRV)	GCV	Ahne (1975)
Perhabdovirus		
Anguillid rhabdovirus		Hill et al. (1980)
Eel virus American	EVA	Sano (1976)
Eel virus European X	EVEX	Sano et al. (1977)
Eel virus C30, B44 and D13	C30, B44, D13	Castric et al (1984)
Perch rhabdovirus	PRV	Dorson et al. (1984)
Pikeperch rhabdovirus		Nougayrede et al. (1992)
Pike rhabdovirus	DK5533	Jörgensen et al. (1993)
Sea trout rhabdovirus		
Brown trout rhabdovirus	ka 903_87	Koski et al. (1992)
Swedish sea trout rhabdovirus	SSTV/ 28/97	Johansson et al. (2001)
Vesiculo-type rhabdoviruses		
Scopthalmus maximus rhabdovirus	SMRV	Zhang et al. (2007)
Siniperca chuatsi rhabdovirus	SCRV	Tao et al. (2008)
Ulcerative disease rhabdovirus	UDRV	Frerichs et al. (1986)
Uncharacterized fish rhabdoviruses		
Rhabdovirus salmonis		Osadchaya & Nakonechnaya (1981)

Genus and species names corresponding to approved virus taxonomy according to the ICTV Master Species List 2012- v1 are in italics.

2.2 THE GENOME AND VIRION PROPERTIES OF NOVIRHABDOVIRUSES AND OTHER FISH RHABDOVIRUSES

The genome of novirhabdoviruses and other fish rhabdoviruses consists of a non-segmented linear, negative-sense single-stranded RNA of about 11 kb in length and encodes five structural rhabdovirus genes in the order 3′-N-P-M-G-L-5′, where N denotes nucleoprotein, P is phosphoprotein, M is matrix protein, G is glycoprotein and L denotes the large protein or a RNA-dependent polymerase. However, novirhabdoviruses have a sixth nonvirion (NV) gene inserted between the G and L genes (Björklund et al. 1996; Hoffmann et al. 2005) (Figure 1). The viral nucleoprotein N, phosphoprotein P, and large polymerase L are essential for RNA synthesis (Conzelmann & Schnell 1994), and the envelope components matrix protein M and glycoprotein G are required for virus release and virus infectivity, respectively (Mebatsion et al. 1996; Mebatsion et al. 1999). The products of the first four genes are major proteins forming enveloped virions. The L gene encodes a multidomain protein, including a putative RNA-dependent RNA polymerase that is virion-associated and mediates replication and expression of the virus genome.

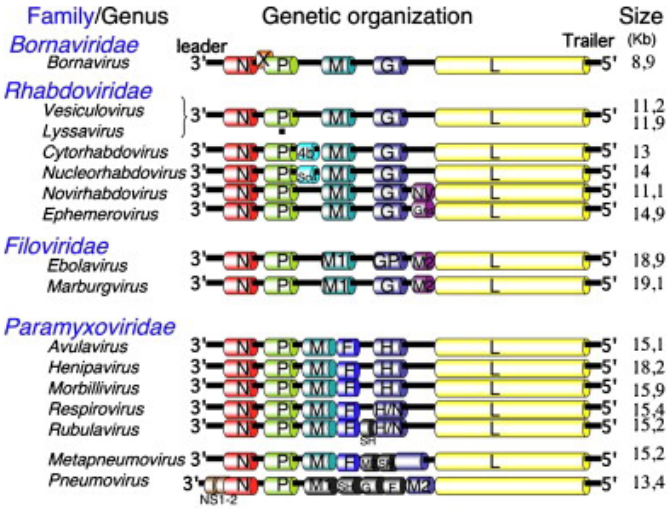

Figure 1. Genetic organization of Mononegavirales. Mononegavirales possess a monopartite genome whose size varies two-fold, from ~9 kb (Bornaviridae) to 19 kb (Filoviridae). The order of the backbone genes from the 3' to the 5' ends of the genome is N–P–M–G–L, where N denotes nucleoprotein, P is phosphoprotein, M is matrix protein, G is glycoprotein and L is the large protein or polymerase. Additional open reading frames, (ORFs) are located between the phosphoprotein and the matrix protein genes and between the glycoprotein and polymerase genes. Large ORFs are indicated by the coloured boxes (adapted from Bourhy et al. 2008). Reprinted from Assenberg et al. (2010) with permission from Elsevier.

Rhabdoviruses, like other Mononegavirales, are enveloped viruses with a lipid bilayer envelope generated from the plasma membrane of the infected host cell. Virions of novirhabdoviruses, perhabdoviruses and vesiculoviruses are bullet-shaped particles that measure approximately 70 nm in width by 180 nm in length.

Extending from the surface of the membrane is one glycoprotein (G) inserted into the envelope that is believed to be important for virus adsorption and attachment to susceptible cells (Jørgensen et al. 1995; Smail & Snow 2011). The M gene encodes the 19 kDa matrix protein believed to act as a bridge between the viral envelope and nucleocapsid in rhabdoviruses (Wagner & Rose 1996), and plays a regulatory role in viral transcription, replication, production and budding in rhabdoviruses (Finke & Conzelmann 2003). The M protein condenses the nucleocapsid and gives the rhabdovirus virion its bullet-shaped appearance (Assenberg et al. 2010). The induction of apoptosis by the M protein causing VHSV has not been identified (Björklund et al. 1997; Du et al. 2004), while the M protein of IHNV induces apoptosis (Chiou et al. 2000).

The N gene encodes 38–41 kDa proteins that are arranged tightly around the viral RNA genome forming the N-RNA complex and playing an important role in the transcription and replication processes by influencing the recognition of transcription signals (Bernard et al. 1992; Bernard et al. 1992; Wagner & Rose 1996). The L protein (157–190 kDa) is thought to perform RNA synthesis as well as mRNA capping and polyadenylation activities (Grdzelishvili et al. 2005; Ogino et al. 2005; Li et al. 2006). The viral RNA-dependent RNA polymerase is a two-subunit complex that consists of a large subunit L (Emerson & Yu 1975) and a non-catalytic cofactor, the phosphoprotein P (Ivanov et al. 2011). They are responsible for the RNA polymerase activity associated with viral particles (Bourhis et al. 2006; Gerard et al. 2009). The G gene encodes the 72–80 kDa major surface glycoprotein antigen located on the envelope surface, and is believed to be important for virus attachment to susceptible cells (Jørgensen et al. 1995; Wagner & Rose 1996).

The NV gene encodes a small non-virion protein that is known only in fish novirhabdoviruses (Nichol et al. 1995; Kurath et al. 1997; Alonso et al. 2004; Thoulouze et al. 2004). It is situated between the glycoprotein (G) and polymerase (L) genes (Tordo et al. 2004). This suggests that there is a relatively low level of evolutionary constraint on the NV gene, although the exact function of the NV protein remains unclear. NV protein of VHSV may play an important role not only in viral replication but also in viral pathogenesis (Kim et al. 2011). According to Ammayappan & Vakharia (2011) and Choi et al. (2011), NV has a role in delaying apoptosis and suppressing the host IFN system. NV has functions in limiting the host IFN response in fish (Choi et al. 2011).

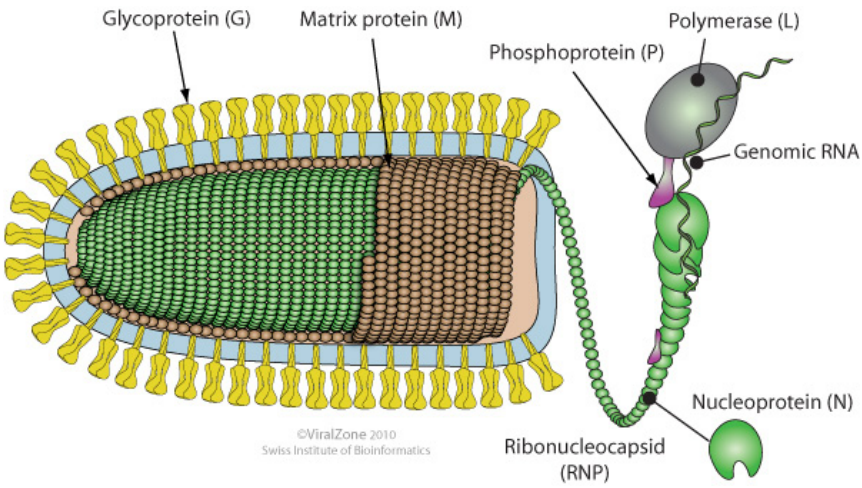

Figure 2. Structure of fish rhabdovirus
http://viralzone.expasy.org/viralzone/all_by_species/76.html
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All rhabdoviruses undergo the same replication cycle. After the first steps of attachment, penetration and uncoating, the nucleocapsid and all the components necessary for early transcription are released into the cytoplasm of the infected cell (Assenberg et al. 2010). Glycoprotein (G) mediates the attachment of the virion, and after attachment, viral particles are endocytosed (Assenberg et al. 2010) (Figure 3).

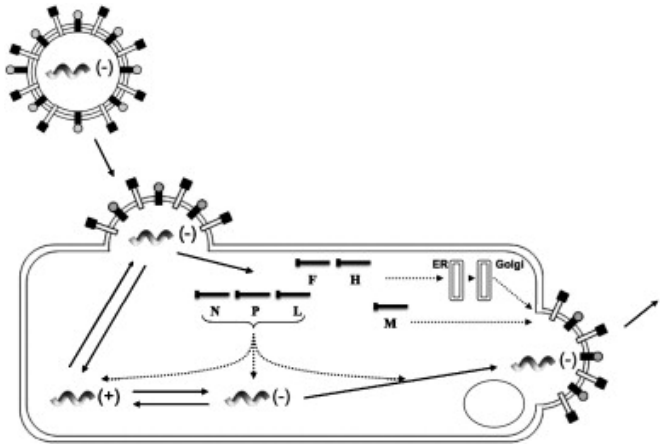

Figure 3. Replication cycle of rhabdoviruses. The replication cycle of all rhabdoviruses the same general steps: attachment, penetration, uncoating, transcription of different genes, translation of different proteins (N, P, M, G and L), viral genome replication, encapsidation and budding. Reprinted from Assenberg et al. (2010) with permission from Elsevier.

2.3 IMMUNITY TO FISH RHABDOVIRUSES

Teleost fish possess the principal components of innate and adaptive immunity. Both types include cellular and humoral responses found in other vertebrates (Magor & Magor 2001; Bone & Moore 2008). Survivors of acute fish rhabdoviral infections develop long-lasting immunity, which is broadly protective against multiple virus strains of the same species (Engelking et al. 1991; Lorenzen et al. 1999). Interactions between the immune system and rhabdoviruses have been well studied for certain fish rhabdoviruses in particular hosts (*i.e.*, IHNV and VHSV in salmonid species), but there are still gaps in our knowledge (Purcell et al. 2012).

2.3.1 INNATE IMMUNITY

Innate immune systems are rapidly induced in response to fish rhabdoviral infection. Replication of fish rhabdoviruses is inhibited by pre-activation of the interferon (IFN) system, regardless of the method used, including the use of IFN-containing supernatants (de Kinkelin & Dorson 1973; de Kinkelin et al. 1982), poly I:C (Eaton 1990), rhabdoviral G protein (LaPatra et al. 2001; Lorenzen et al. 2002; Verjan et al. 2008) or recombinant IFN (Wang et al. 2006; Ooi et al. 2008). Fish rhabdoviral infection also results in rapid IFN protection and an IFN-stimulated gene (ISG) response that correlates with virus levels in the tissues. This does not necessarily correlate with protection (Purcell et al. 2004; Penaranda et al. 2009; Purcell et al. 2009). Although rhabdoviruses are sensitive to the effects of IFN, virulent rhabdoviruses can continue to replicate owing to the abilities of the matrix (M) protein to mediate host-cell shutoff and the non-virion (NV) protein to subvert programmed cell death and suppress functional IFN (Purcell et al. 2012). VHSV resistance in trout is correlated with *ex vivo* replication of VHSV in fin tissues, implicating epidermal tissues as pivotal in the anti-VHS defence system (Quillet et al. 2001; Quillet et al. 2007). Host–virus dynamics at virus entry points (*i.e.*, the fins; Harmache et al. 2006) may help limit the internal spread of viral infection by alerting systemic sites via IFNs and other cytokines.

Cell-mediated cytotoxicity (CMC) consists of specific and nonspecific CMC in fish (Fischer et al. 2006; Nakanishi et al. 2011). The nonspecific cytotoxic cells (NCC) and NK-like cells (Evans & Jaso-Friedmann 1992; Shen et al. 2003; Shen et al. 2004) are responsible for nonspecific CMC. Basic research is needed to definitively identify the cells that contribute to key cellular effector functions in fish (Purcell et al. 2012).

2.3.4 ADAPTIVE IMMUNITY

The adaptive immune system is an important component of the anti-rhabdoviral immunity that very specifically recognizes viral antigens. Adaptive immune recognition is mediated by two types of antigen receptor: T-cell receptors and B-cell receptors that very specifically recognize viral antigens (Medzhitov 2007). The organization of lymphoid tissues, where lymphocytes develop, encounter antigen and become activated, profoundly differs between fish and mammals (Flajnik & Du Pasquier 2008). While B lymphopoiesis occurs in bone marrow in mammals, fish lack both bone marrow and lymph nodes, and B cells differentiate in the anterior kidney or pronephros, with B and T cell responses occurring mainly in spleen and mucosal territories (Huttenhuis et al. 2005; Zwollo et al. 2005). Many features of T cell immunity are similar between fish and mammals (Castro et al. 2013). However, fish B cells lack efficient affinity maturation (Hofmann et al. 2010).

Neutralizing antibodies induced by infection and/or vaccination are critical components of long-term adaptive immunity to fish rhabdoviruses (Lorenzen & LaPatra 1999). Passive immunization with sera containing neutralizing antibodies protects fish, even when titers fall below detectable levels (Hershberger et al. 2011) Neutralizing antibodies are unlikely to play a role in surviving the acute infection phase in coldwater fish species, since neutralizing antibodies are not typically detectable until several weeks post-infection (Purcell et al. 2012).

Rhabdoviral persistence is related to suppression of the adaptive immune response, but it is not clear whether individuals become long-term carriers or if the virus persists by cycling in the population among naïve and/or convalescent hosts (Hershberger et al. 2010b).

2.4 THE IMPACTS OF EMERGING FISH DISEASES CAUSED BY RHABDOVIRUSES

The emerging fish viral diseases affect livelihoods locally, and many have also impacted on regional or national economies, with their environmental impacts being both direct and indirect. Rhabdoviruses have the most serious socio-economic impact on aquaculture, especially the novirhabdoviruses VHSV and IHNV in trout and other salmonids and the vesiculovirus SVCV in cyprinids. Disease can impact directly on wild populations and the ecosystem by changing host abundance and predator/prey populations, reducing genetic diversity and causing local extinctions (Arthur & Subasinghe 2002). VHSV in the North American Great Lakes has also resulted in massive mortalities and spread to at least 25 native freshwater fish species with the potential for similar broader environmental impacts (Walker & Winton 2010). Viral diseases of fish have occurred through expansion of the known geographic range via the natural movement of infected hosts, vectors or carriers and

with subsequent exposure of naive, and often highly susceptible species (Walker & Winton 2010). A single introduction of VHSV resulted in infection of the most susceptible species, such as the native muskellunge, *Esox masquinongy*, and the invasive round gobi, *Neogobius melanostomus*, which could amplify the virus sufficiently to increase the infection pressure on a broad range of naive species within the larger ecosystem (Groocock et al. 2007).

VHSV causes mortality rates as high as 90% (Olesen 1998; Snow et al. 1999), resulting in a large economic threat to the aquaculture industry (Nylin & Olesen 2001). In Poland, up to 10% of the total production of rainbow trout, estimated to be around 12 000 tonnes, has been lost in some years because of VHS (Reichert et al. 2013).

2.5 DETECTION AND ISOLATION OF FISH RHABDOVIRUSES

The "gold standard" for the detection of fish viruses is isolation of the virus in cell culture followed by its immunological or molecular identification. The OIE Manual of Diagnostic Tests for Aquatic Animals includes several methods for detecting IHNV, SVCV and VHSV (OIE 2012b), and the sampling plans and diagnostic methods for the detection and confirmation of VHS and IHN are laid down in Commission Decision 2001/183/EC (European Commision 2001). Serological methods involving polyclonal and monoclonal antibodies are used for both diagnostics and research. Molecular techniques such as DNA probes, polymerase chain reaction (PCR) assays and quantitative polymerase chain reaction (qPCR) assays have been developed for confirmation of the diagnosis, identification, quantification and investigating the molecular epidemiology of the most significant rhabdoviruses affecting fish. In many regions of the world, the majority of all cultured fish stocks are surveyed on a regular basis for viral pathogens.

2.6 VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUS VHSV

VHSV causes viral haemorrhagic septicaemia (VHS), also known as Egtved disease, which is a serious rhabdovirus disease of rainbow trout and is listed as a notifiable disease by the World Organisation for Animal Health (Office International des Epizooties, OIE), as well as being a listed disease in the EU. It afflicts over 80 species of fish, mainly farmed rainbow trout, farmed turbot, farmed Japanese flounder, *Paralichthys olivaceus*, and a broad range of wild freshwater and marine species (Meyers & Winton 1995; Mortensen et al. 1999; Skall et al. 2005a; Elsayed et al. 2006; Lumsden et al. 2007; EFSA 2008; Jonstrup et al. 2009; Emmenegger et al. 2011) in several parts of the northern hemisphere.

Because VHSV is very heterogeneous in nature, a number of different sero- and genotypes can be distinguished by the use of monoclonal antibodies in the neutralization test or ELISA, or by restriction fragment length polymorphism RFLP analysis or sequencing. Based on plaque neutralization assay (PNT) results, three serotypes have been recognized: type 1 is primarily represented by the F1 strain (isolated from Denmark); type 2 is represented by the Hededam isolate from Danish rainbow trout (Jørgensen & Meyling 1972; Jørgensen 1980); and type 3 is represented by a French brown trout isolate (Einer-Jensen et al. 2002).

VHSV has been divided into four major genotypes (I, II, III, IV) with further subtypes of genotypes I (Ia–Ie) and IV (IVa–IVc) that correlate with the geographic location (Benmansour et al. 1997; Stone et al. 1997; Nishizawa et al. 2002; Thiery et al. 2002; Einer-Jensen et al. 2004; Snow et al. 2004; Pierce & Stepien 2012).

VHS was considered a continental European freshwater disease, but following the isolation of VHSV from a broad range of free-living fish species in the North Atlantic Ocean (Mortensen et al. 1999; Smail 2000), in the North Pacific Ocean (Brunson et al. 1989; Hopper 1989; Meyers et al. 1992; Nishizawa et al. 2002; Lumsden et al. 2007; Al-Hussinee et al. 2011) and in the North American Great Lakes (Lumsden et al. 2007; Al-Hussinee et al. 2011), the geographical distribution appeared to be much wider. It was hypothesized that VHSV had become established in European freshwater sites in the early days of the industry when it was common practice to use untreated trash marine fish in the feed (Dixon 1999; Skall et al. 2005a). Genetic links have been found between these marine viruses and those isolated from fish in European freshwater sites (Benmansour et al. 1997; Stone et al. 1997; Einer-Jensen et al. 2004; Snow et al. 2004).

2.6.1 VHS DISEASE

2.6.1.1 Signs and pathological changes

The clinical signs and outcome of infection vary depending on the VHSV genotype, fish species, age, stress level, temperature and other environmental factors (VHSV Expert Panel and Working Group 2010). Common acute clinical signs are skin darkening, anaemia, exophthalmia, and epidermal haemorrhages, sometimes with ulceration (King et al. 2001b; Einer-Jensen et al. 2006). The gills are markedly pale, reflecting generalized anaemia (Smail & Snow 2011). Rainbow trout fry are the most susceptible to disease because the target organs represent most of the body weight (Smail & Snow 2011). The presence of ascites and petechiae scattered in the peritoneum and musculature is also a typical symptom of VHSV infection (Wolf 1988) (Figure 4). VHSV-infected fish suffer severe tissue alterations such

as necrotic degeneration of the kidneys, spleen, liver and intestine (Yasutake & Rasmussen 1968; Brudeseth et al. 2002; Kim & Faisal 2010c; Kim & Faisal 2010a; Kim & Faisal 2010b; Al-Hussinee et al. 2011; Kim & Faisal 2011). In the rainbow trout, the endothelial cells of the kidney, liver and spleen are the target organs of the VHS virus, causing widespread haemorrhagic changes within 1–4 days of infection (Yasutake & Rasmussen 1968; Evensen et al. 1994; Brudeseth et al. 2002; Smail & Snow 2011). Typical histological changes are focal to multifocal degeneration and necrosis of a variety of tissues, although the pattern varies between species and virus genotypes (Marty et al. 1998; Isshiki et al. 2001; Brudeseth et al. 2005; Lumsden et al. 2007), and there can also be a vasculitis (Wolf 1988; Lumsden et al. 2007).

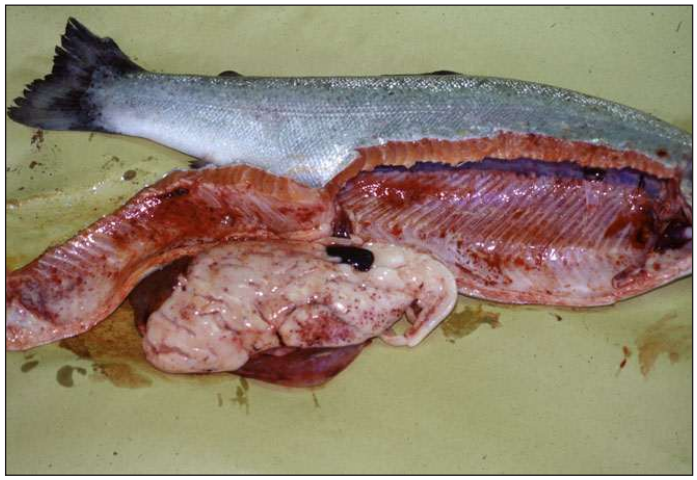

Figure 4. Acute VHS causing haemorrhaging in the viscera and musculature (Picture Eija Rimaila-Pärnänen, Evira).

Although VHS is termed a haemorrhagic disease, the degree of haemorrhage is generally limited and it is more common to find degenerative changes and necrosis of tissues (Wolf 1988). Mortality of 80–100% due to virulent strains is normal at the optimum temperature. Mortality is typically about 10–50% and the nervous chronic state is often seen with fingerlings and growers (Smail & Snow 2011).

2.6.1.2 Forms of disease

VHS occurs in acute, subacute and chronic forms followed by a carrier state in surviving fish in rainbow trout (Wolf 1988; Smail & Snow 2011). In some hosts, the chronic form is characterized by nervous manifestations (Wolf 1988; Smail & Snow 2011). The clinical signs and pathological changes vary depending on the disease

form, with the acute disease being the quickest and resulting in greater mortality. Chronically infected fish experience a prolonged course with low mortality and often resulting in carrier fish, which shed VHSV into the surrounding environment (Neukirch 1985). Over the course of a VHS outbreak, the tissue tropism transitioned from an acute systemic phase to a chronic phase associated with neurological disease in which fish cleared the virus from all tissues except the nervous system (Lovy et al. 2012). A chronic and neurological form of VHS has previously been suggested in Pacific herring (Hershberger et al. 2010b) and rainbow trout (Ghittino 1965). In rainbow trout, persistent VHSV infections occurred in the brain, which lasted up to 379 days following experimental waterborne infection (Neukirch & Glass 1984; Neukirch 1986). Erratic swimming behaviour characterized as spiralling, flashing, a corkscrewing motion and surface swimming are seen in the nervous form (Wolf 1988; Smail & Snow 2011).

2.6.1.3 Transmission

VHSV primarily infects a new host via excreta by horizontal transmission (Neukirch 1985). Chronically infected fish often become carriers and shedders of VHSV. Viral shedding via the urine has been demonstrated in clinical diseases in rainbow trout (Neukirch & Glass 1984; Neukirch 1985). The virus attaches to both the gill epithelium and skin (Chilmonczyk et al. 1995), and viral replication has been shown in skin and gills, especially in pillar cells (Neukirch 1984; Yamamoto et al. 1992). The fin bases and skin are also the portal of entry into fish (Harmache et al. 2006). VHSV can additionally be detected from the skin after fish have become viraemic (Yamamoto et al. 1992; Brudeseth et al. 2002; Harmache et al. 2006; Brudeseth et al. 2008; Montero et al. 2011).

Traditional insights into VHSV pathogenesis very much suggest infection from fish to fish through urine as the only means of transmitting virus (Kim & Faisal 2011). Oral transmission has, however, been demonstrated by the feeding of infected fish (Wolf 1988; Schönherz et al. 2012a). The transportation of VHSV has been shown to be possible via diverse vectors, including boating, ballast water, fishing tackle, and animals such as amphipod crustaceans, leeches, turtles and birds (Faisal & Schulz 2009; Bain et al. 2010; Faisal & Winters 2011; Goodwin & Merry 2011b). Fish-to-fish transmission appears prevalent via shed mucus and urine, which probably increases in spawning aggregations (Winton & Einer-Jensen 2002).

Because VHSV is a highly transmissible virus, it can be transmitted by intraperitoneal injection, bathing and cohabitation in experimental studies (Castric & de Kinkelin 1980; de Kinkelin & Castric 1982; Meier et al. 1994; Follett et al. 1997; Kocan et al. 1997; Snow et al. 2000; King et al. 2001a; Bowden 2003; Muroga et al. 2004; Skall et al. 2004; Snow et al. 2005; Hershberger et al. 2007; Lopez-Vazquez

et al. 2007; Al-Hussinee et al. 2010; Hershberger et al. 2010a; Kim & Faisal 2010a; Kim & Faisal 2012).

2.6.1.4 Predisposing factors

Several factors such as genetics, age, species, stress and environmental variables influence the susceptibility of fish hosts to VHS (Hedrick et al. 2003; Skall et al. 2005a). VHSV is most likely to occur at temperatures from 9–12 °C (Wolf 1988; Dopazo et al. 2002; Hedrick et al. 2003; Kim & Faisal 2010a). VHSV replicates best in cell lines at 14–15 °C (de Kinkelin & Scherrer 1970). The pathogenicity of VHSV decreases at or above 20 °C (de Kinkelin & Scherrer 1970). The temperature range of VHSV genotype IVb appears to be same as published values for VHSV genotype I, which has an optimum in the range of 9–12 °C and an upper limit of 18–20 °C (Goodwin & Merry 2011a). According to Vestergård-Jørgensen (1982), temperature was inversely correlated with the longevity of the infection in rainbow trout: the infection was persistent for 14 weeks at 5 °C, 2 weeks at 10 °C and undetectable at 20 °C. VHSV can survive up to 14 days in freshwater but only 4 days in seawater (Hawley & Garver 2008). The VHSV infection persisted for 300–400 days in rainbow trout at 4 °C (Neukirch 1985). According to Hedrick et al. (2003), temperatures and virulences may show differences among VHSV genotypes. Infected fish of fry to juvenile age may experience up to 100% mortality, while fish that are older when infected may exhibit 25–75% mortality rates (Meyers & Winton 1995; Kocan et al. 1997; Skall et al. 2005a).

2.6.2 GEOGRAPHIC DISTRIBUTION AND HOST RANGE OF VHSV

VHSV infection was first discerned in European salmonid aquaculture in the 1930s (Schärperclaus 1938; Rasmussen 1965; Jørgensen 1980). A similar syndrome was also reported to occur in Poland (Pliszka 1946), and in the early 1950s in Denmark and France (Besse 1955; Rasmussen 1965; Jørgensen 1980) and also in Bavaria, which afflicted grayling and whitefish (Reichenbach-Klinke 1959). The virus was isolated (isolate DK-F1) from infected rainbow trout in 1962 (Jensen 1965; Einer-Jensen et al. 2004). VHSV was reported to have also appeared in Italy, Czechoslovakia, Switzerland and Norway (Ghittino 1965; Zwillenberg et al. 1965; Håstein et al. 1968; Tesarcik et al. 1968). In addition, Pastoret et al. (1976) carried out isolations in Belgium and Meier & Pfister (1981) reported on an acute outbreak of VHS among pike fry in Switzerland. It has since been identified across the Northern Hemisphere, occurring in marine/estuarine/fresh waters of continental Europe (Meyers & Winton 1995; Benmansour et al. 1997; Thompson et al. 2011).

Until 1988, VHSV was recognized as a virus only affecting freshwater fish species (Skall et al. 2005a), although VHSV isolations from farmed rainbow trout kept in marine net pens had occurred in Europe before 1988 (Castric & de Kinkelin 1980; Hørlyck et al. 1984). The first isolate of marine VHSV was made from Atlantic cod with the ulcus syndrome in the Baltic Sea (Jensen et al. 1979), and was identified as VHSV in 1987 (Jørgensen & Olesen 1987). This virus was similar to other tested northern European marine isolates without mortality to rainbow trout (Skall et al. 2004). VHSV has not been isolated from Atlantic salmon in European waters during routine monitoring for VHSV, including thousands of samples collected in Norway, the UK and Ireland (Skall et al. 2005a), but it has been isolated from Spain (Jimenez de la Fuente, J. et al. 1988). Isolations of VHSV from marine fish, combined with VHS outbreaks in turbot farms in Germany in 1991 (Schlotfeldt et al. 1991), Scotland in 1994 (Ross et al. 1994) and Ireland in 1997 (Skall et al. 2005a) led to sampling of wild marine fish for virological investigation. VHSV was isolated from herring, sprat, cod and four-beard rockling from the Baltic Sea (Mortensen et al. 1999), and from Atlantic herring in the English Channel (Dixon et al. 1997). VHSV is endemic in the Baltic Sea, Kattegat/Skagerrak, the North Sea and around the British Isles (Skall et al. 2005a). In the Baltic Sea, VHSV is mainly found in herring and sprat, whereas Norway pout is the predominant host in the North Sea (Hedrick et al. 2003; Skall et al. 2005b). None of the fish sampled during the Danish, Norwegian, Scottish and English cruises showed pathology typical of VHS in rainbow trout (Mortensen et al. 1999; King et al. 2001b; Brudeseth & Evensen 2002; Dixon et al. 2003; Skall et al. 2005b). In infection trials using turbot aged less than one year, many isolates were found to be virulent (King et al. 2001a).

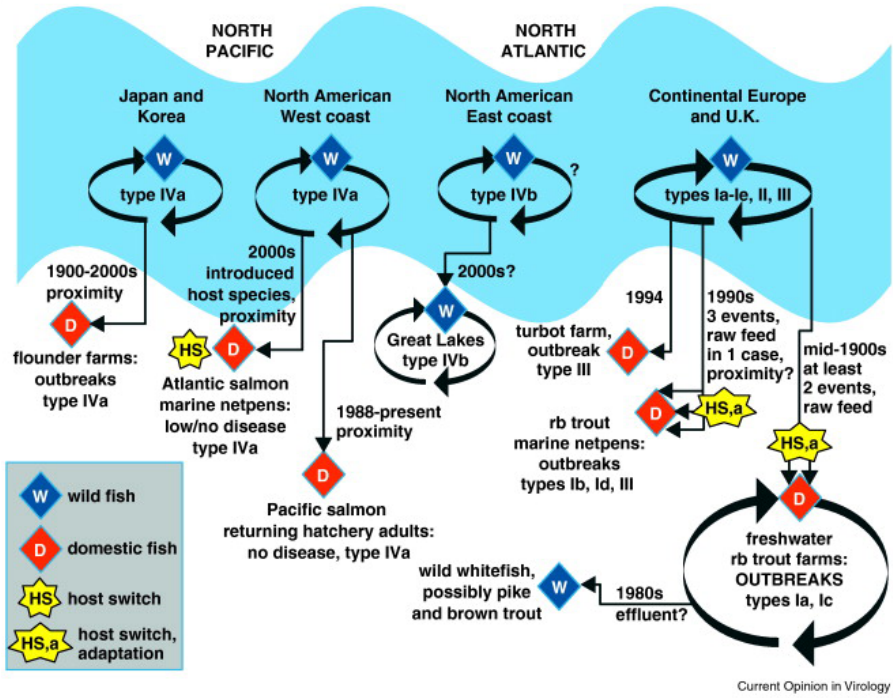

Figure 5. Documented transmissions of VHSV across the interface between wild and domestic host fish populations. The blue shape represents the extensive reservoir of VHSV among diverse marine fish species. Circular arrows indicate established endemic virus replication cycles, with genetic type(s) of VHSV indicated where known. In most cases, genotyping has provided evidence for the probable direction of flow of the viruses in the transmission events shown. Straight or rectangular arrows indicate the direction of virus transmission events, with estimated year(s) and hypothesized mechanism(s) written beside each arrow. The figure shows six cases of transmission involving spillover from wild to domestic hosts, and one spillback event from domestic to wild fish. 'rb trout' denotes rainbow trout (Meier et al. 1986; Meier et al. 1994; Stone et al. 1997; Nordblom & Norell 2000; Isshiki et al. 2001; Nishizawa et al. 2002; Snow et al. 2004; Skall et al. 2005a; Dale et al. 2009; Kim et al. 2009). Reprinted from Kurath & Winton (2011) with permission from Elsevier.

Prior to the late 1980s, VHSV was believed to be restricted to continental Europe. In 1988, a new VHSV strain IV (now classified as IVa) was isolated for the first time in North America, from ascending chinook (Hopper 1989) and coho salmon (Brunson et al. 1989) (Figure 5). The North American strain of VHSV was isolated from farmed Atlantic salmon in the late 1990s and 2002 in British Columbia, Canada (Amos & Thomas 2002). All types of VHSV are generally non- to low pathogenic for Atlantic salmon by immersion infection (de Kinkelin & Castric 1982; Traxler et al. 1999; King et al. 2001a), but VHSV may be a potential problem in Atlantic salmon farming (Skall et al. 2005a). It was also identified in Pacific cod, sardine, mackerel, smelt, three-spined stickleback, mummichog, Greenland halibut and pilchards from Alaska, California and Oregon in the USA and from British Columbia

and Newfoundland in Canada (Traxler et al. 1999; Amos & Olivier 2001; Dopazo et al. 2002; Olivier 2002; Hedrick et al. 2003). VHSV genotype IVa is believed to have an extensive reservoir and cause mass mortalities in the Northeast Pacific Ocean in wild marine fishes, including Pacific herring, Pacific hake and walleye pollock (Meyers & Winton 1995; Meyers et al. 1999; Hedrick et al. 2003). In 2003, VHSV was isolated first time from the North American Great Lakes, from muskellunge from Lake St. Clair (Michigan and Ontario) (Elsayed et al. 2006). Within six years, VHSV spread to different parts of the Great Lakes (Groocock et al. 2007; Lumsden et al. 2007).

The geographic range of VHSV expanded to Asia with isolation from Japanese olive flounder and black seabream in marine waters off Japan in 1996 and Korea in 1999 (Isshiki et al. 2001; Kim et al. 2003; Kim & Faisal 2010b; Kim & Faisal 2011; Studer & Janies 2011) (Figure 5). VHSV has been isolated from both farmed and wild flounder (Takano et al. 2000; Nishizawa et al. 2002) and also farmed black rockfish (Isshiki et al. 2003), oblong rockfish, Japanese jack mackerel, red sea bream and cultured and wild Pacific sandeel (Watanabe et al. 2002). The North American type of VHSV is virulent to Japanese flounder (Isshiki et al. 2001), and it has also been isolated from cultured, diseased Japanese flounders in Korea (Kim et al. 2003).

Species susceptible to VHSV include herring, whitefish, pike, haddock, Pacific cod, Atlantic cod, Pacific salmon, rainbow trout, rockling, brown trout, turbot, sprat, grayling and olive flounder (European Commision 2006a; European Commision 2012). VHSV has been isolated from over 80 different freshwater and marine fish species during the past two decades throughout Europe, North America and Asia (Table 2). The most susceptible species is rainbow trout to genotype Ia, but VHSV also causes mortality in farmed turbot and Japanese flounder (Schlotfeldt et al. 1991; Ross et al. 1994; Isshiki et al. 2001; Kim et al. 2009). In the Great Lakes region of the USA and Canada, severe VHSV genotype IVb outbreaks involving many fish species have been observed (Meyers & Winton 1995; Meyers et al. 1999; Hedrick et al. 2003; Skall et al. 2005a). North American VHSV isolates and the northern European marine isolates are less pathogenic to rainbow trout than the European freshwater strains (Meyers et al. 1994; Follett et al. 1997; Skall et al. 2004). Australia, Chile, New Zealand and South Africa, where salmonid agriculture is practised in the southern hemisphere, are currently free from VHSV (Skall et al. 2005a).

Table 2. Fish species for which there is conclusive evidence of susceptibility to VHSV (Skall et al. 2005b; EFSA 2008; OIE 2012a). Latin names of fish species are not repeated elsewhere in this chapter.

Order	Family	Common name	Latin name
Salmoniformes (Salmons)	Salmonidae (salmonids)	Rainbow trout	*Oncorhynchus mykiss*
		Steelhead trout	
		Chinook salmon	*Oncorhynchus tshawytscha*
		Coho salmon	*Oncorhynchus kisutch*
		Golden trout*[2]	*Oncorhynchus aguabonita*
		Chum salmon*[3]	*Oncorhynchus keta*
		Sockeye salmon*[3]	*Oncorhynchus nerka*
		Atlantic salmon	*Salmo salar*
		Brown trout	*Salmo trutta*
		Lake trout*[1]	*Salvelinus namaycush*
		Brook trout*[1]	*Salvelinus fontinalis*
		Grayling	*Thymallus thymallus*
		Whitefish	*Coregonus spp.*
		Whitefish*[1]	*Coregonus lavaretus*
		Lake whitefish	*Coregonus clupeaformis*
Esociformes	Esocidae	Muskellunge	*Esox masquinongy*
		Northern pike	*Esox lucius*
Clupeiformes	Clupeidae	Atlantic herring	*Clupea harengus*
		Pacific herring	*Clupea pallasii*
		European sprat	*Sprattus sprattus*
		South American pilchard/Sardine	*Sardinops sagax*
		American gizzard shad	*Dorosoma cepedianum*
Gadiformes (cod)	Gadidae	Atlantic cod	*Gadus morhua*
		Pacific cod	*Gadus macrocephalus*
		Haddock	*Melanogrammus aeglefinus*
		Poor cod	*Trisopterus minutus*
		Norway pout	*Trisopterus esmarkii*
		Blue whiting	*Micromesistius poutassou*
		Whiting	*Merlangius merlangus*
		Alaska Pollock	*Theragra chalcogramma*
		Pacific tomcod	*Microgadus proximus*
	Lotidae (hakes)	Fourbeard rockling	*Enchelyopus cimbrius*
		Burbot	*Lota lota*
	Merlucciidae	(North) Pacific hake	*Merluccius productus*
Pleuronectiformes (flatfish)	Pleuronectidae	Dab	*Limanda limanda*
		Flounder	*Platichthys flesus*
		European plaice	*Pleuronectes platessa*
		English sole	*Parophrys vetula*

Table 2. continued

Pleuronectiformes (flatfish)	Pleuronectidae	Greenland halibut	*Reinhardtius hippoglossoides*
		Marbled flounder*[2]	*Pleuronectes yokohamae*
		Atlantic halibut*[1]	*Hippoglossus hippoglossus*
	Scophthalmidae	Turbot	*Scophthalmus maximus*
	Paralichthyidae	Japanese flounder	*Paralichthys olivaceus*
	Soleidae	Senegalese sole	*Solea senegalensis*
Siluriformes (catfish)	Ictaluridae (North American freshwater catfish)	Brown bullhead	*Ictalurus nebulosus*
		Channel catfish	*Ictalurus punctatus*
Osmeriformes	Argentinidae	Lesser argentine	*Argentina sphyraena*
	Osmeridae (smelts)	Eulachon/Smelt	*Thaleichthys pacificus*
		Surf smelt	*Hypomesus pretiosus*
Peciformes (perch-like)	Ammodytidae	Pacific sand lance	*Ammodytes hexapterus*
		Sandeel	*Ammodytes spp.*
		Pacific sandeel	*Ammodytes personatus*
	Gobiidae	Sand goby	*Pomatoschistus minutus*
		Round goby	*Neogobius melanostomus*
	Embiotocidae	Shiner perch	*Cymatogaster aggregata*
	Centrarchidae (sunfish)	Largemouth bass	*Micropterus salmoides*
		Smallmouth bass	*Micropterus dolomieu*
		Bluegill	*Lepomis macrochirus*
		Black crappie	*Pomoxis nigromaculatus*
		Rock bass	*Ambloplites rupestris*
		Pumpkinseed	*Lepomis gibbosus*
		Freshwater drum	*Aplodinotus grunniens*
	Percidae (perches)	Yellow perch	*Perca flavescens*
		Walleye	*Sander vitreus*
	Scombridae	Chub mackerel, Pacific mackerel	*Scomber japonicus*
	Moronidae (temperate basses)	White bass	*Morone chryso*
		Striped bass	*Morone saxatilis*
		White perch	*Morone americana*
	Sparidae	Gilthead seabream	*Sparus aurata*
		Black porgy*[2]/ Black sea bream	*Acanthopagrus schlegelii*
	Moronidae (temperate bass)	European seabass*[1]	*Dicentrarchus labrax*
		Red seabream*[2]	*Pagrus major*

Table 2. continued

Peciformes (perch-like	Carangidae	Japanese amberjack*[2]	*Seriola quinqueradiata*
	Serranidae	Hong Kong grouper*[2]	*Epinephelus akaara*
Scorpaeniformes (scorpionfish and flatheads)	Anoplopomatidae (sablefish)	Sablefish	*Anoplopoma fimbria*
	Sebastidae (Rockfish, rockcod and thornyheads)	Black rockfish Mebaru (Japanese)	*Sebastes inerrmis*
		Schlegel's black rockfish*[2]	*Sebastes schlegelii*
Anguilliformes	Anguillidae	European eel	*Anguilla anguilla*
Cyprinodontiformes	Fundulidae	Mummichog	*Fundulus heteroclitus*
Gasterosteiformes	Gasterosteidae	Three-spined stickleback	*Gasterosteus aculeatus*
	Aulorhynchidae	Tube-snout	*Aulorhynchus flavidus*
Cypriniformes (carp)	Catostomidae	Silver redhorse	*Moxostoma anisurum*
		Shorthead redhorse	*Moxostoma macrolepidotum*
	Cyprinidae (minnows or carp)	Bluntnose minnow	*Pimephales notatus*
		Emerald shiner	*Notropis atherinoides*
		Spottail shiner	*Notropis hudsonius*
		Iberian nase	*Chondrostoma polylepis*
		Zebra danio*[2]	*Danio rerio*
Percopsiformes (troutperch, pirate perch and cavefish)	Percopsidae (troutperch)	Trout-perch	*Percopsis omiscomaycus*
Petromyzontiformes (lamprey)	Petromyzontidae (lamprey)	Lamprey (sea)	*Petromyzon marinus*

*[1] Infection trial, immersion; *[2] Infection trial, IP injection; *[3] No virus isolation, PCR only

2.6.3 GENETIC DIVERSITY AND MOLECULAR EPIDEMIOLOGY OF VHSV

VHSV isolates have been divided into four major phylogenetic groups (genotypes I–IV) based on the nucleoprotein (N) and glycoprotein (G) gene ORF sequences from over 70 isolates (Einer-Jensen et al. 2004; Snow et al. 2004). It has been identified across the Northern Hemisphere, with genotypes I–III predominantly occurring in marine/estuarine/fresh waters of continental Europe and genotype IV in North America and Asia. Genotypes seem to be more associated with the geographical regions of isolation than with fish species or pathogenicity (Snow et al. 1999; Einer-Jensen et al. 2004; Lumsden et al. 2007). Strain I is classified into five substrains: Ia–Ie. Ia is endemic to freshwater European aquaculture, infects 13 fish species, and has the most known haplotypes. Ib occurs in marine/estuarine waters of the Baltic and North Seas and infects 10 fish species. Substrain Ie occurs in the marine/estuarine Black Sea in brown trout and turbot (Nishizawa et al. 2006).

- Genotype I (Ia–Ie): European freshwater VHSV isolates, marine isolates from the Baltic Sea and freshwater and marine isolates from Georgia and Turkey;
- Genotype II: Marine isolates from the Baltic Sea;
- Genotype III: Isolates from the North Sea, Skagerrak and Kattegat;
- Genotype IV (IVa–IVc): North American and Asia isolates.

Genotypes I (a–d) include a wide range of virus strains originating from brackish water and freshwater rainbow trout farms in Europe (Benmansour et al. 1997; Stone et al. 1997; Snow et al. 1999; Thiery et al. 2002; Einer-Jensen et al. 2004). Most VHSV isolates causing outbreaks in European rainbow trout farms cluster in sublineage Ia, of which isolates have been reported from most continental European countries (Einer-Jensen et al. 2004; Snow et al. 2004; Stone et al. 2008; Toplak et al. 2010). However, genotype Ia isolates have also been detected in brown trout, pike and grayling (Meier et al. 1994; Jonstrup et al. 2009). Almost all genotype Ia isolates have caused outbreaks in freshwater farms, but have also been isolated from rainbow trout in seawater net pens and from turbot (Schlotfeldt et al. 1991; Snow et al. 2004). According to Kahns et al. (2012), there are two distinct clades within sublineage Ia, namely Ia-1 and Ia-2. VHSV Ia-1 isolates mainly originate from infected Danish freshwater catchments and a few outbreaks in Germany and the UK, whereas trout isolates originating from other continental European countries cluster in clade Ia-2 (Kahns et al. 2012). Certain genotype I isolates from marine fish species from different parts of the Baltic Sea, the Skagerrak and Kattegat, and the isolate from the English Channel have shown the same ancestral source, as they all belong to genotype Ib (Einer-Jensen et al. 2004). In Sweden, an outbreak of VHSV genotype Ib has been observed in marine cultured rainbow trout farms (Nordblom 1998; Nordblom & Norell 2000). Some Danish rainbow trout isolates from earlier dates cluster in genotype Ic (Kahns et al. 2012), while some old Danish isolates are grouped together in genotype Id with an old Norwegian isolate from the 1960s (Kahns et al. 2012). Genotype Ie includes isolates from both the freshwater and the marine (Black Sea) environment in Georgia and Turkey (Einer-Jensen et al. 2004; Nishizawa et al. 2006).

Genotype II includes marine isolates found from Baltic herring and sprat in the Baltic Sea (Snow et al. 1999; Einer-Jensen et al. 2004). Genotype III has been isolated in several fish species such as Atlantic cod, Atlantic herring, sprat and other fish species from the North Sea (Einer-Jensen et al. 2004; Snow et al. 2004), the North Atlantic (Lopez-Vazquez et al. 2006b) and seawater-reared rainbow trout in Western Norway (Dale et al. 2009).

Genotype IV isolates from North America and Asia are divided into two sublineages, IVa and IVb (Skall et al. 2005a), but isolates found on the Atlantic coast of North America have tentatively been placed in a new subgroup, IVc (Pierce

& Stepien 2012). Sublineage IVa is primarily restricted to the marine environment in both North America and Asia (Meyers & Winton 1995; Nishizawa et al. 2002; Hedrick et al. 2003; Lee et al. 2007), and is believed to have an extensive reservoir in wild marine fishes in the Northeast Pacific Ocean (Meyers & Winton 1995; Meyers et al. 1999; Hedrick et al. 2003). Lately, it has expanded to Asia with its isolation from Japanese olive flounder and black seabream in marine waters off Japan in 1996 and Korea in 1999 (Kim et al. 2009; Studer & Janies 2011). Thus, all Japanese isolates are genotype IVa (Ito et al. 2004), except one genotype Ia isolate from farmed Japanese flounder (Nishizawa et al. 2002; Einer-Jensen et al. 2005). Sublineage IVb isolates have only been observed in the Great Lakes region and marine systems in North America (Gagne et al. 2007; Kane-Sutton et al. 2010; Thompson et al. 2011). Additionally, IVb has been isolated from a leech (*Myzobdella lugubris*) and an amphipod (*Diporeia* spp.), which may serve as invertebrate vectors (Faisal & Winters 2011).

2.6.4 DETECTION AND IDENTIFICATION OF VHSV

The following techniques are used for the diagnosis of VHS:

A. Conventional virus isolation with subsequent serological virus identification;
B. Virus isolation with simultaneous serological virus identification;
C. Other diagnostic techniques (IFAT, ELISA, RT-PCR).

The confirmation of the first case of VHS in farms in approved zones must not be based on method C alone. Either method A or B must also be used. Detailed recommendations for VHSV diagnostic methods are provided in the manuals of EURL and OIE (EURL-Fish 2010; OIE 2012a).

2.6.4.1 Clinical methods

The occurrence of the signs and gross pathology findings described in section 2.6.1.1 should lead to extended clinical examination for VHS. The haematocrit (HCT) is very low in the acute phase of VHS and the blood appears light red and transparent (OIE 2012a). Immunohistochemistry reveals VHSV-positive endothelial cells, primarily in the vascular system (Evensen et al. 1994).

2.6.4.2. VHSV isolation in cell culture

During and immediately following an outbreak, virus can be isolated readily in cell culture (Wolf 1988). The inoculation of fish cell lines with fish tissues processed for virus isolation is the "gold standard" for surveillance programmes (for detecting carrier fish). The tissue material to be examined to yield the highest viral titres includes spleen, anterior kidney, and either heart or encephalon. When sampling farms with broodstocks, ovarian fluid may be examined (EURL-Fish 2010; OIE 2012a). Organ samples from a maximum of 10 fish may be pooled (EURL-Fish 2010; OIE 2012a). Fish cell lines such as Bluegill fry (BF-2), rainbow trout gonad (RTG-2), epithelioma papulosum cyprinid (EPC), fathead minnow (FHM), Chinook salmon embryo (CHSE-214), snakehead fish (SSN-1) and pike gonad (PG) (Wolf 1988) are susceptible to VHSV. BF-2 and RTG-2 are the most sensitive cell lines for freshwater isolates from rainbow trout (Olesen & Jørgensen 1992; Lorenzen et al. 1999). The most common method in Europe for the isolation of VHSV from wild marine fish has been inoculation of fish tissue onto BF-2 cells (Dixon et al. 1997; Mortensen et al. 1999; King et al. 2001b; Brudeseth & Evensen 2002), but EPC, FHM and CHSE-214 cells have also been used (Dixon et al. 1997; Smail 2000). Cell susceptibility is ranked in the order BF-2, FHM, RTG-2 and EPC (Lorenzen et al. 1999). EPC pretreated with polyethylene glycol may be more sensitive to several genotype IV isolates (Brunson et al. 1989; Hopper 1989; Meyers et al. 1992; Meyers et al. 1994; Marty et al. 1998; Hershberger et al. 1999; Meyers et al. 1999; Traxler et al. 1999; Hedrick et al. 2003). Inoculated cell cultures are incubated at 15°C for 7–10 days and inspected regularly (at least three times a week) for the occurrence of viral cytopathic effect (CPE) at 40 to 150 x magnification (EURL-Fish 2010; OIE 2012a). Subcultivation is performed if no CPE has developed similar to that of primary cultivation. The cell lineage and passage number can have a marked effect on virus replication (McAllister 1997). In carrier (clinically healthy) fish, the isolation of VHSV can be problematic (Skall et al. 2005a) as false negative results may occur.

2.6.4.3 Immunochemical and protein-based methods

If evidence of CPE has been observed in a cell culture, the medium (supernatant) is collected and examined by one or more techniques. Standard detection methods for VHSV are based on isolation in cell culture followed by virus-specific confirmation if evidence of CPE has been observed. The virus can be identified using a plaque neutralisation test (PNT) or a microtitre neutralization test (Smail & Snow 2011). Viral antigen can be detected with an immunofluorescent antibody test (IFAT), but it is time consuming and sometimes not as sensitive as desired (Miller et al. 1998; Lorenzen et al. 1999; Einer-Jensen et al. 2002). The use of MAb IP5B11 (Lorenzen

et al. 1988) is recommended as the reference reagent, as all VHSV isolated to date react with this MAb.

The antigen-capture enzyme-linked immunosorbent assay (ELISA) is widely used for detection of virus in cell culture supernatants. The first layer in the ELISA is the polyclonal catching antibody to VHSV, which may be a MAb. A blocking reaction follows to coat unadsorbed solid-phase sites, and virus is then added. The next step is the addition of MAb, normally to G protein, and then the addition of an antispecies antibody to MAb, which is tagged with an enzyme (Smail & Snow 2011). VHSV ELISA for a polyclonal direct antigen-capture system (Way & Dixon 1988) and three models of ELISA for virus detection have been described: indirect ELISA, direct ELISA and antigen-capture ELISA, using a variety of MAbs to the viral glycoprotein G (Mourton et al. 1990).

Serology has not been accepted as a routine diagnostic method for assessing the virus status in fish populations, although the detection of fish antibodies is a good indicator of previous infection (Lorenzen & LaPatra 1999). ELISA, IFAT or 50% PNT can be used to detect such antibodies, and ELISA has proven to be more sensitive and less time and material consuming than either IFAT or 50% PNT (Olesen et al. 1991).

2.6.4.4 Molecular techniques

Molecular tests (RT-PCR and real-time PCR) have become common because of their rapidity and sensitivity. The reverse transcriptase polymerase chain reaction (RT-PCR) and nested PCR have proven to be useful tools for fish rhabdovirus diagnosis and molecular epidemiology, and these are currently considered the most sensitive methods for fish rhabdovirus detection (Bruchhof et al. 1995; Miller et al. 1998; Strømmen & Stone 1998; Guillou et al. 1999; Lopez-Vazquez et al. 2006a). Because of the primary position of the N gene in the VHSV genome, N transcripts are the most abundant during viral infection, and the N gene has therefore been the target of several molecular assays used to detect VHSV (Batts et al. 1993; Chico et al. 2006; Lopez-Vazquez et al. 2006a; Matejusova et al. 2008; Cutrín et al. 2009; Hope et al. 2010). The RT-PCR amplification of VHSV G genes was found to be a simple, highly specific and sensitive method, allowing the differential diagnosis of VHS (Bruchhof et al. 1995; Miller et al. 1998; Chico et al. 2006; Chico et al. 2009; Cutrín et al. 2009).

Chico et al. (2006) developed a TaqMan PCR procedure for VHSV tested with one genotype I strain. Matejusova et al. (2008) also reported the development of this methodology for the diagnosis of VHSV strains, although it was only tested on European strains, and not on American isolates. Cutrin et al. (2009) developed two real-time PCR protocols (based on SYBR Green and TaqMan®) applied to cell culture

for the detection of strains belonging to all known genotypes of VHSV. The use of real time PCR, mainly the SYBR Green format, is recommended for the diagnosis of VHSV because of its sensitivity, specificity, repeatability and reproducibility (Cutrín et al. 2009). Real-time reverse transcriptase quantitative PCR (RT-qPCR) is a method for the detection of microorganisms in a research setting and is widely accepted (Mackay 2004). Carver et al. (2011) developed the VHSV RT-qPCR to provide universal detection of all genotypes of VHSV. Four genotype-specific RT-qPCR arrays were designed to amplify a region of the N gene for European genotypes (Ia, Ib, II and III) (Bland et al. 2013).

2.6.5 EPIDEMIOLOGY, PREVENTION AND CONTROL

In the absence of anti-viral agents, control methods for VHS currently rely on official health surveillance schemes coupled with control measures. Examples of practices that have been successful in reducing the number of infected farms in an endemic area and preventing reinfection include stamping-out and fallowing procedures (Olesen & Korsholm 1997; Olesen 1998). In the first place, a way by which the spread of VHSV can be prevented is by proper disinfection of contaminated equipment and holding areas and ultraviolet irradiation of incoming water (Øye & Rimstad 2001). Chlorine, hypochlorite, formalin, sodium hydroxide and iodophors are effective disinfectants (Wolf 1988; Smail & Snow 2011). Increasing the temperature above 20 °C (Parry & Dixon. 1997) or dilution in seawater (Kurita et al. 2002) are also used to inactivate VHSV. However, according to Hawley & Garver (2008), VHSV could remain stable in either water type regardless of its environmental origin.

Research on vaccine development for VHS has been ongoing for more than three decades, but no accepted commercial vaccine is yet available. Candidate vaccines have included inactivated vaccines, attenuated live vaccines, a recombinant vaccine in prokaryotic and eukaryotic expression systems, and DNA-based vaccines. DNA-based vaccines have proven to be very promising, inducing good protection against VHS disease (Lorenzen & LaPatra 2005).

2.7 PERHABDOVIRUS

Historically, fish rhabdoviruses were first assigned to a genus based on their morphology, protein profiles after gel electrophoresis and serological cross-reactivity (Hoffmann et al. 2005; Kuzmin et al. 2006). The genus *Vesiculovirus* comprises an ecologically diverse but genetically similar group of viruses. Vesicular stomatitis Indiana virus (VSIV), which can infect insects, cattle, horses and pigs, is the prototype of the genus *Vesiculovirus*. The *Spring viraemia of carp virus* (SVCV) was first

isolated from carp in Yugoslavia in the early 1970s (Fijan et al. 1971). Until 1981, SVCV and pike fry rhabdovirus (PFRV) were the only known vesiculo-type viruses responsible for disease epizootics in cultured fishes. Later, SVCV was classified as a member of the genus *Vesiculovirus*. The classification of PFRV as a vesiculovirus is tentative (Carstens 2010). However, there are significant differences between SVCV and other vesiculoviruses, such as the lower replication temperature range of fish rhabdoviruses than mammalian rhabdoviruses. Therefore, two new genera of fish rhabdoviruses based on phylogenetic analysis, *Sprivivirus* and *Perhabdovirus*, have recently been suggested by the ICTV *Rhabdoviridae* study group. It is proposed to place both SVCV and PFRV into a new genus, *Sprivivirus* (ICTV 2013c). The primary hosts for SVCV and other viruses in the genus *Sprivivirus* are freshwater fish species within the order Cypriniformes (ICTV 2013c). The genus *Perhabdovirus* is already approved by ICTV (ICTV 2013b).

The genus *Perhabdovirus* comprises three species: *Perch rhabdovirus* (PRV), *Anguillid Rhabdovirus* (AngRV) and *Sea trout rhabdovirus* (STRV) (Table 1). The representative isolates are perch rhabdovirus (PRV), Anguillid rhabdovirus isolates eel (*Anguilla anguilla* and *Anguilla rostrata*) virus European X (EVEX) and eel virus American (EVA), and both brown trout rhabdovirus ka903_87 (ka903_87 or LTRV 903/87) and Swedish sea trout virus isolate 28/97 (SSTV 28/97) (ICTV 2013b). Siniperca chuatsi rhabdovirus (SCRV) (Tao et al. 2008), isolated from mandarin fish or Chinese perch (*Siniperca chuatsi*), shares some similarity with perhabdoviruses, but has a gene encoding a small protein between the M and G genes that is not present in PRV, EVEX or ka903_87. Because of this finding and the relatively low level of nucleotide sequence identity with PRV, EVEX and ka903_87, SCRV will not be considered a member within the genus *Perhabdovirus* until more information is available. PRV infects the broadest range of fish species, and of the three proposed species within the new genus, PRV is likely to pose the greatest threat to both cultivated and wild fish populations (ICTV 2013b).

In 1974, a rhabdovirus was isolated from a young American eel imported from Cuba to Japan and tentatively designated eel virus American (EVA) (Sano 1976). In 1976, another rhabdovirus was isolated in a shipment of European elvers from France to Tokyo, which was named eel virus European X (EVEX) because of its European origin (Sano 1976). EVA and EVEX are morphologically, serologically, physicochemically and genetically highly similar, and regarded as two strains of a single virus species (Hill et al. 1980). The tentative classification of EVEX in the genus *Vesiculovirus* is still based on electron microscopy (Sano et al. 1977) and the PAGE (polyacrylamide gel electrophoresis protein) profile (Hill et al. 1980), as no molecular data have been available so far. Since the first isolation, EVEX has been detected in wild and farmed European eels originating from many countries, including Denmark, Germany, France, Italy, the Netherlands, Sweden and the United Kingdom (Galinier et al. 2012). The EVEX leader sequence possesses

additional nucleotides, as do other tentatively assigned fish vesiculo-like viruses, but the untranscribed intergenic sequences are slightly longer than those of established vesiculovirus species such as ka903/87, STRV and SCRV belongs to a clade close to the vesiculoviruses (Galinier et al. 2012). According to Galinier et al. (2012), SVCV and PFRV are positioned at the interface of this clade and the mammalian vesiculoviruses, while SMRV seems to be more basal, and the idea of creating a new clade close to the genus *Vesiculovirus*, and regrouping some fish vesiculo-like viruses, was thus to be supported.

In the early 1970s, after the isolation of SVCV, another vesiculo-type virus was isolated and characterised: pike fry rhabdovirus (PFRV), the causative agent of red disease of pike (de Kinkelin et al. 1973). Until 1981, PFRV and SVCV had been the only known vesiculo-type viruses responsible for disease epizootics in cultured fishes. A rhabdovirus isolated from perch failing to grow in the EPC cell line and pathogenic for perch was reported from individuals from France exhibiting a loss of equilibrium and swimming disturbances in 1980 (Dorson et al. 1984). It was antigenically distinct from the other then known fish rhabdoviruses (Dorson et al. 1984). This virus was designated as perch rhabdovirus and was first demonstrated to be pathogenic to its host species only after intracranial inoculation of cell culture-grown virus. Later, however, perch rhabdovirus was found to produce septicaemic infections in juvenile pike with mortality via bath challenge (Dorson et al. 1987). In northern Finland, the brown trout rhabdovirus virus ka903_87 (also called lake trout rhabdovirus 903/87, LTRV or montavirus) was isolated from farmed brown trout, *Salmo trutta* m. *lacustris*, in 1987 (Koski et al. 1992; Jørgensen et al. 1993).

There were no other recorded outbreaks associated with perhabdovirus until 1990, when another rhabdovirus was isolated that was antigenically similar to perch rhabdovirus and caused mortality to the larvae of pikeperch, *Sander lucioperca* (previously *Stizostedion lucioperca*), at a pond fish farm in central France (Nougayrede et al. 1992). A rhabdovirus was also isolated in 1990 from diseased grayling in a salmonid fish farm in eastern France (Betts et al. 2003). A little later, a rhabdovirus (DK5533) was isolated from asymptomatic pike in Denmark (Jørgensen et al. 1993). This virus, which displayed a protein electropherogram typical of rhabdoviruses belonging to the genus *Vesiculovirus*, was found to be antigenically similar to perch rhabdovirus in immunofluorescence assays and was experimentally pathogenic for juvenile pike by waterborne infection (Jørgensen et al. 1993). Antisera raised against DK5533 were also shown to cross-react with the brown trout rhabdovirus virus (ka 903/87) (Jørgensen et al. 1993). In 1995, a vesiculo-type rhabdovirus was isolated from perch and largemouth bass originating from the same farming site as that which had given rise to the pikeperch rhabdovirus in France (Betts et al. 2003). Both isolates were neutralised by a trout antiserum to perch rhabdovirus (Betts et al. 2003). In 1996, the Swedish sea trout virus (SSTV) was isolated from sea trout, *Salmo trutta trutta*, from the archipelago of Stockholm,

Sweden, and it was shown to be genetically related to the brown trout rhabdovirus (Johansson et al. 2002). Rhabdovirus was isolated from perch in Norway in 1997 (Dannevig et al. 2001). According to Betts et al. (2003), the perch and largemouth bass isolates from 1995 and the grayling isolate from 1990 were antigenically related to the 1990 pikeperch isolate and the 1981 perch isolate, but the biochemical and genetic relationships of these viruses with each other and the PFRV, SVCV and trout isolates remained unclear.

Despite the increasing impact of perhabdoviruses, their diversity has been poorly investigated. Perhabdoviruses can propagate in BF- 2, EPC and RTG-2 cell lines at 15 °C (Betts et al. 2003). The gross clinical and pathological characteristics of the disease cases have been characterized, and the viruses can be characterized by the study of viral morphology, antigenicity, protein composition, and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) based on L, N and G genes (Björklund et al. 1994; Betts et al. 2003; Talbi et al. 2011). The perch rhabdoviruses can be further characterized by immunofluorescence (IF) and a plaque neutralization test using a panel of different antisera against several fish rhabdoviruses (Dannevig et al. 2001). Based on partial N and complete G gene analysis, perch rhabdovirus was divided into four genogroups, A, B, D and E, by Talbi et al. (2009). Perch rhabdoviruses were reported to be a threat to farmed and natural percid populations (Jørgensen et al. 1993; Dannevig et al. 2001; Betts et al. 2003). Their control is impeded by a lack of specific diagnostic tools, among them PCR. There is no administrative pressure to develop diagnostic tools, because perch rhabdovirus is not listed by the EU. Additional data on genetic diversity, transmission routes and alternative hosts for perch rhabdoviruses are needed to understand their evolution (Talbi et al. 2011). The control of perch rhabdovirus relies on the disinfection of fish egg strings, strict biosecurity measures and minimizing stress, because there are no vaccines or medical treatments for the virus (Aquaplan 2013).

3 AIMS OF THE STUDY

This study was conducted to investigate the epidemiology of the fish rhabdoviruses isolated in Finland.The specific aims of the study were as follows:

1. To study the epidemiology of the VHSV strains isolated from rainbow trout farms in Finland by comparing the nucleotide sequences of the G and NV genes (I).

2. To study the virulence of VHSV genotype Id isolated from rainbow trout *Oncorhynchus mykiss* and VHSV genotype II isolated from the European river lamprey, *Lampetra fluviatilis,* by infection trial in rainbow trout (I, II)

3. To clarify the role of the European river lamprey in the epidemiology of VHSV (II).

4. To clarify the role of wild fish especially, Baltic herring, *Clupea harengus membras* L., in the epidemiology of VHSV in brackish water (III).

5. To identify and characterise the rhabdovirus isolated from diseased grayling *Thymallus thymallus* (IV).

4 MATERIALS AND METHODS

4.1 SAMPLING OF FISH (I, II, III, IV)

VHSV was diagnosed for the first time in Finland in 2000 from four rainbow trout farms in brackish water in Pyhtää and Åland. VHSV had been isolated from 24 farms in three separate locations by the end of 2004: Åland and Uusikaupunki in the Baltic Sea and Pyhtää in the Gulf of Finland (I). The surveillance and sampling for VHSV carried out in Finland during 2000–2012 is summarized in Table 3.

Table 3. Surveillance testing for VHSV in fish by Evira in the years 2000–2011.

Year	Inland farm/samples[1]	Marine* farm/samples[1]	LRL[2]/ samples[1]
2000	85/576	73/443	45/392
2001	72/549	85/472	44/360
2002	72/464	61/489	38/224
2003	73/520	54/236	46/314
2004	77/487	58/244	49/347
2005	75/541	68/256	43/356
2006	73/471	55/205	32/232
2007	81/450	83/288	35/298
2008	69/440	43/154	32/278
2009	73/318	51/177	27/209
2010	65/3726	53/2890	31/3096
2011	44/2588	38/1256	31/2017
2012	68/5406	49/1332	-

[1] The number of pools in 2000–2009; since 2010, the number of fish. One pool comprises samples of about 10 fish.
[2] LRL= natural feeding pond programme, both hatcheries and ponds.These are situated almost exclusively in inland waters.
*Marine is brackish water in Finland

Altogether 2621 healthy adult river lampreys were caught during the spawning migration from Finnish rivers draining into the Gulf Bothnia during 1999–2008 (II). A total of 7580 Baltic herring without clinical signs of disease were obtained directly from local fisherman or with the help of the staff of the Finnish Game and Fisheries Research Institute (III). Details of the samples of herring are summarized in Table 4.

Table 4. Locations, dates and catch methods for Baltic herring sampled for virus isolation. The symbol " Δ " after the sampling site indicates that the sample originated from the coast, whereas " O " indicates samples collected from the open sea.

Sample	Sampling site	Sampling date	Co-ordinates N	E	Catch method	No. samples
1	Archipelago Sea Δ	11.5.2004	60°25	22°05	trap net	31
2	Archipelago Sea Δ	19.5.2004	60°26	22°06	trap net	35
3	Archipelago Sea Δ	25.5.2004	60°27	21°56	trap net	28
4	Archipelago Sea Δ	1.6.2004	60°14	22°25	trap net	18
5	Archipelago Sea Δ	2.6.2004	60°26	22°05	trap net	26
6	Archipelago Sea O	9.6.2004	60°29	21°29	trap net	30
7	Bothnian Sea O	10.11.2004	61°55	18°30	midwater trawl	30
8	Archipelago Sea O	26.11.2004	60°46	20°11	midwater trawl	36
9	Bothnian Sea O	10.12.2004	60°50	18°30	midwater trawl	23
10	Bothnian Sea O	14.12.2004	61°50	20°40	midwater trawl	33
11	Archipelago Sea O	8-9.2.2005	60°48	20°10	midwater trawl	36
12	Bothnian Sea O	1.3.2005	61°07	20°18	midwater trawl	36
13	Bothnian Sea O	22.3.2005	61°45	20°30	midwater trawl	36
14	Bothnian Sea O	5.4.2005	61°15	20°30	midwater trawl	36
15	Archipelago Sea O	25.4.2005	60°52	20°40	midwater trawl	36
16	Archipelago Sea Δ	25.5.2005	60°34	21°44	open ended trap net	36
17	Archipelago Sea Δ	27.5.2005	60°24	22°08	trap net	36
18	Bothnian Sea O	27.4.2006	61°40	21°05	midwater trawl	36
19	Bothnian Sea O	11.5.2006	61°01	18°30	midwater trawl	24
20	Archipelago Sea Δ	17.5.2006	59°55	23°02	trap net	36
21	Bothnian Sea O	18.5.2006	63°09	20°52	midwater trawl	36
22	Gulf of Finland O	31.5.2006	60°27	26°42	trap net	24
23	Archipelago Sea Δ	1.6.2006	60°24	22°06	trap net	36
24	Gulf of Finland O	7.6.2006	60°23	26°42	open ended trap net	24
Total						758

Grayling fry samples (13 in total from two inland farms) were investigated for rhabdovirus (IV). The farms were situated in different catchment areas in Finland. The virus was also isolated from wild perch and sea trout caught from Baltic Sea (IV).

4.2 VIRUS ISOLATION IN CELL CULTURE (I, II, III, IV)

Virus isolation was attempted from organ samples (EURL-Fish 2010). Pooled heart or brain, anterior kidney, spleen (lamprey do not have a spleen as a single organ and this was not therefore sampled from them) and brain tissue from 10 fish/

lampreys (= 1 sample) 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 BF-2 (Wolf et al. 1966) and EPC (Fijan et al. 1983) in 24-well dishes (Nunc A/S) in final dilutions of 1:100 and 1:1000. The inoculated cultures were incubated in a CO_2 incubator (buffered with sodium carbonate 7.5%, Gibco 25080) and without CO_2 from 2009 onwards, at 16°C and inspected regularly under a microscope for the occurrence of cytopathic effect (CPE). After 7 days of incubation at 16 °C, supernatant from samples without CPE was diluted 1:100 and 1:1000, sub-cultured onto fresh cells and incubated for a further 7 days. When CPE was observed, the supernatant was collected and stored at -70 °C for future studies.

4.3 IDENTIFICATION OF VIRUSES (I, II, III, IV)

4.3.1 DETECTION OF VHSV (I, II, III, IV)

4.3.1.1 Enzyme-linked immunosorbent assay (ELISA) (I, II, III, IV)

Aliquots of 50 µl of culture medium from cell cultures showing evidence of CPE were analysed with a commercial ELISA kit according to the manufacturer's instructions (Test-Line Ltd., Brno, Czech Republic) to test for the presence of VHSV. The principles of the ELISA test are described in chapter 2.6.4.3.

4.3.1.2 Indirect fluorescent antibody test (IFAT) (I, II)

The indirect fluorescent antibody test (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 was 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 of 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), infectious pancreatic necrosis virus (IPNV) or/ and uninfected cells as negative controls.

4.3.2 DETECTION OF OTHER VIRUSES: IHNV, IPNV, SVCV (I, II, IV)

4.3.2.1 Enzyme-linked immunosorbent assay (ELISA) (I, II, III)

Aliquots of 50 µl of culture medium from cell cultures showing evidence of CPE were analysed with a commercial ELISA kit according to the manufacturer's instructions to test for the presence of IPNV and SVCV (Test-Line Ltd., Brno, Czech Republic) and IHNV (Bio-X Diagnostics S.P.R.L. , Jemelle, Belgium). The principles of the ELISA test are described in chapter 2.6.4.3.

4.3.3 DETECTION OF PERCH RHABDOVIRUS (IV)

4.3.3.1 Indirect fluorescent antibody test (IFAT) (IV)

The IFAT technique was performed as described previously in chapter 4.3.1.2. except that the polyclonal antibody (PAb) against the rabbit anti-perch rhabdovirus F28 (provided by the European Union Reference Laboratory for Fish Diseases, EURL, National Veterinary Institute, Technical University, Aarhus, Denmark) and rabbit anti-brown trout rhabdovirus ka903_87 (Monta 3005-2) (EURL, Århus, Denmark) were diluted in phosphate-buffered saline and applied as the first antibody on the monolayer. FITC-labelled swine anti-rabbit immunoglobulin was used as the second antibody (DAKO A/S). Brown trout rhabdovirus ka903_87 was used as a positive control and VHSV and uninfected cells as negative controls.

4.3.4 POLYMERASE CHAIN REACTION (PCR) AND SEQUENCING (I, II, III, IV)

4.3.4.1 VHSV (I, II, III)

For nucleotide sequence analysis, total RNA was extracted from 200 µl (I) or 140 µl (II ,III) of viral supernatant from virus-infected cells using RNA affinity spin columns (RNeasy Total RNA kit; Qiagen GmbH, Hilden, Germany) (I) or the QIAamp® Viral RNA Mini Kit (Qiagen) (II,III) according to the manufacturers instructions and stored until use at -70 °C. The typing of the strains and confirmation of the results was carried out by using a reverse transcriptase-polymerase chain reaction (RT-PCR) test specific for VHSV. To produce material for sequencing and genetic typing of the strains, primers of various primer sets were prepared (Table 5). They were designed on the basis of the published genomic sequences and sequence data generated in these studies. The generated PCR products were purified using Microspin S-400 HR Columns (Amersham Biosciences) according to the manufacturer's instructions. PCR products were visualised by gel electrophoresis and sequenced using both the forward and reverse primer. Sequencing was performed with the ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems) using the Big Dye v3.1 Cycle Sequencing Kit (Applied Biosystems) The raw sequence data were analysed using Sequencing Analysis Software 5.1 (Applied Biosystems).

4.3.4.2 Perch rhabdovirus (IV)

The typing of the strains and confirmation of the results was carried out by using a reverse transcriptase-polymerase chain reaction (RT-PCR) assay specific for perhabdovirus. RNA was isolated from infected cell cultures using the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was generated either separately or in a one-step PCR reaction. The reaction mix for the separate cDNA synthesis contained 2.5 µM random primers (Random Hexamers, Applied Biosystems, Foster City, CA, USA), 2 µl 10x buffer II (Applied Biosystems), 2 mM dNTPs (Applied Biosystems), 2.5 mM $MgCl_2$, 20 units of RNase Inhibitor (Applied Biosystems), 150 units of MuLV Reverse Transcriptase (Applied Biosystems) and 5 µl of RNA. The reactions were incubated at 37 °C for 90 min and stored at -70 °C until further use.

Several primers were used to amplify fragments of viral glycoprotein (G) and RNA polymerase (L) genes (Table 5). They were designed based on published sequences (Betts et al. 2003; Talbi et al. 2011) and sequences obtained in this study. The PCR conditions were the following: an initial pre-heating step of 10 min at 95 °C, 25 cycles of 94 °C for 1 min, 50 °C for 2 min and 72 °C for 2 min, followed by a final

extension step of 15 min at 72 °C. With primers oPVP116 & oPVP118 and oPVP126 & Rha-G-seqR2, the QIAGEN Onestep RT-PCR Kit was used with 5 µl of RNA as a reaction template. The one-step PCR protocol was the following: cDNA synthesis at 50 °C for 30 min, deactivation of reverse transcriptase and activation of DNA polymerase at 95 °C for 15 min, 35 cycles of 94 °C for 30 s, 42 °C for 30 s and 72 °C for 1 min, followed by a final extension step of 7 min at 72 °C. PCR products were visualized, sequenced and raw sequence data were analysed as described in chapter 4.3.2.1.

Table 5. Primers used in this study for PCR amplification and sequence analysis

Virus	Primer*	Sequence (5''→3')	Publication
VHSV	G1+	CGGGCAGGCGAAGGACTA	I, II, IIII
	G2+	ATGGAATGGAATACTTTTTTC	I, II, IIII
	G3+	CAACCTCGCCCTGTCAAACTCAT	I, II, IIII
	G4+	GGGGGCGCGAGGAAACTG	I
	G5+/GLF+	TGGACCCGGCAAGGCACACT	I, II, IIII
	NV+	CCCGGACATCGAGAAGTATCAGAG	I
	G1-	CGGAGACGCTGGTGACTGATA	I, II, IIII
	G2-	TGTGATCATGGGTCCTGGTG	I, II, IIII
	G3-	GTCCCCAAATATCATCCCATCGTA	I, II, IIII
	G4-	TCAGACCGTCTGACTTCTGGA	I
	NAH-	CTAGGAGACTTATCCTCATGTC	II, IIII
	NV-	GCCAAGCGCCCGAAGAGC	I
Perch rhabdovirus	GF+	TGTCCDATRCAHGGARATYT	IV
	GL-	THTBATCTGYKAKCCAAA	IV
	LF+	CTCTGATGTCATGGGAAYTG	IV
	LR-	CATCCCAACAGACAAGCYTGTC	IV
	oPV116	ACWTGTGAYTWCMGWTGGTATGG	IV
	oPV118	CTGTTAGCTGTTTTTTTCATA	IV
	oPV126	GATATGAAAAAAACTGCAACAG	IV
	Rha-G-seqR2	GAGGAGTCCTCTATGTTGGTC	IV

*Forward primers marked +, reverse primers marked -

4.4 ANALYSIS OF SEQUENCE DATA (I, II, III, IV)

To determine the similarity of VHSV (I, II, III) and grayling (IV) isolates with other published VHSV (I, II, III) and fish rhabdovirus (IV) isolates, a nucleotide similarity search (BLAST) was performed via www.ncbi.nlm.nih.gov and a number of representative VHSV isolates from each genotype (I, II, III) (summarised in Table 6) and fish vesiculovirus and vesiculo-type/perhabdovirus isolates (IV) were selected to further analyse the genetic relationships. The multiple sequence alignments were performed with the MegAlign program using the Clustal Method (DNASTAR) and

ClustalW2 software (Larkin et al. 2007). Phylogenetic and molecular evolutionary analyses were conducted using the neighbour-joining DNA distance method with the Kimura 2-parameter algorithm in Mega version 3.0 (Kumar et al. 2004) (I), MEGA version 4 (Tamura et al. 2007) (II, III) and MEGA version 5 (IV) (Tamura et al. 2011). A phylogenetic tree was constructed from G (I, II, III) and G-NV (I) gene alignments using neighbour- joining DNA distance method (I), the maximum parsimony DNA distance method determined by 1000 data set bootstrap resampling within the MEGA program (II, III), and from the partial L gene with the maximum likelihood method determined by 1000 data-set bootstrap resampling within the MEGA 5 program. The pairwise sequence divergences were calculated using the MegAlign program of LASERGENE, with default settings (II, III, IV). The fish rhabdovirus isolates used in this study for phylogenetic analysis with details of isolates retrieved from GenBank are presented in Table 6.

Table 6. The fish rhabdovirus isolates used for phylogenetic analysis with isolate details retrieved from GenBank.

Isolate code	Year of isolation	Origin	Host species	Virus (genotype)	Accession No	Publication
DK-F1	1962	Denmark	Rainbow trout	VHSV (I)	AF345857	I, II, III
NO-A163-68 EG46	1968	Norway*	Rainbow trout	VHSV (Id)	AY546621	I, II, III
FR-07-71	1971	France	Rainbow trout	VHSV (Ia)	AY546616	I, II, III
DK-Hededam	1972	Denmark	Rainbow trout	VHSV (I)	Z93412	I, II, III
DK-M.Rhabdo	1979	Denmark	Cod	VHSV (Ib)	Z93414	I, II, III
GE-1.2	1981	Georgia	Rainbow trout	VHSV (Ie)	AY546619	II
FR-02-84	1984	France	Rainbow trout	VHSV (Ia)	U28800	II
DK-3592B	1986	Denmark	Rainbow trout	VHSV (Ia)	AY356632	I
DK-5131	1988	Denmark	Rainbow trout	VHSV (Ic)	AF345859	II, III
DK-5151	1988	Denmark	Rainbow trout	VHSV (Ia)	AF345858	II
96-43	1996	English Channel	Herring	VHSV (Ib)	AF143862	I, II, III
DK 1p40	1996	Baltic Sea	Rockling	VHSV (Ib)	AY546575	II
DK-200070-4	2000	Denmark	Rainbow trout	VHSV (Ia)	AY546612	I,II,III
SE-SVA-1033	2000	Kattegat	Rainbow trout	VHSV (Ib)	AY546623	I, II,III
FiA01.00**	2000	Åland, Finland	Rainbow trout	VHSV (Id)	AY546614	I, II,III
FiP01.00	2000	Pyhtää, Finland	Rainbow trout	VHSV (Id)	AM086355	I, II,III
FiP02.00	2000	Pyhtää, Finland	Rainbow trout	VHSV (Id)	AM086356	I
FiP03.00	2000	Pyhtää, Finland	Rainbow trout	VHSV (Id)	AM086357	I
FiA02a.01	2001	Åland, Finland	Rainbow trout	VHSV (Id)	AM086358	I
FiA02b.01	2001	Åland, Finland	Rainbow trout	VHSV (Id)	AM086359	I
FiA03.01	2001	Åland, Finland	Rainbow trout	VHSV (Id)	AM086360	I
FiA04.01	2001	Åland, Finland	Rainbow trout	VHSV (Id)	AM086361	I
FiA05.01	2001	Åland, Finland	Rainbow trout	VHSV (Id)	AM086362	I
FiP04.01	2001	Pyhtää, Finland	Rainbow trout	VHSV (Id)	AM086363	I
FiP03.01	2001	Pyhtää, Finland	Rainbow trout	VHSV (Id)	AM086364	I
FiA03.02	2002	Åland, Finland	Rainbow trout	VHSV (Id)	AM086365	I
FiA04.02	2002	Åland, Finland	Rainbow trout	VHSV (Id)	AM086366	I
FiA06.02	2002	Åland, Finland	Rainbow trout	VHSV (Id)	AM086367	I

Isolate code	Year of isolation	Origin	Host species	Virus (genotype)	Accession No	Publication
FiA07.02	2002	Åland, Finland	Rainbow trout	VHSV (Id)	AM086368	I
FiA08.02	2002	Åland, Finland	Rainbow trout	VHSV (Id)	AM086369	I
FiA09.02	2002	Åland, Finland	Rainbow trout	VHSV (Id)	AM086370	I
FiA10.02	2002	Åland, Finland	Rainbow trout	VHSV (Id)	AM086371	I
FiA11.02	2002	Åland, Finland	Rainbow trout	VHSV (Id)	AM086372	I
FiA12.02	2002	Åland, Finland	Rainbow trout	VHSV (Id)	AM086373	I
FiA13.02	2002	Åland, Finland	Rainbow trout	VHSV (Id)	AM086374	I
FiA14.02	2002	Åland, Finland	Rainbow trout	VHSV (Id)	AM086375	I
FiA15.02	2002	Åland, Finland	Rainbow trout	VHSV (Id)	AM086376	I
FiA16.02	2002	Åland, Finland	Rainbow trout	VHSV (Id)	AM086377	I
FiA17.02	2002	Åland, Finland	Rainbow trout	VHSV (Id)	AM086378	I
FiA03.03	2003	Åland, Finland	Rainbow trout	VHSV (Id)	AM086379	I
FiA18.03	2003	Åland, Finland	Rainbow trout	VHSV (Id)	AM086380	I
FiU01.03	2003	Uusikaupunki, Finland	Rainbow trout	VHSV (Id)	AM086381	I,II,III
FiA03.04	2004	Åland, Finland	Rainbow trout	VHSV (Id)	AM086382	I
FiA19.04	2004	Åland, Finland	Rainbow trout	VHSV (Id)	AM086383	I
NO- CH.15.02.08	2008	Norway	Herring	VHSV (Ib)	FJ384761	III
1p52	1996	Baltic Sea	Sprat	VHSV (II)	AY546576	I,II,III
1p53	1996	Baltic Sea	Herring	VHSV (II)	AY546577	I,II,III
1p55	1996	Baltic Sea	Sprat	VHSV (II)	AY546578	II,III
FI-lamprey-743.03	2003	Finland, Lestijoki	Lamprey	VHSV (II)	GQ504013	II,III
ka363_04-ka365_04	2004	Archipelago Sea	Herring	VHSV (II)	HQ112198-HQ112200	III
FI-ka366-04	2004	Archipelago Sea	Herring	VHSV (II)	HQ112234	III
ka367_04-ka369_04	2004	Archipelago Sea	Herring	VHSV (II)	HQ112201-HQ112203	III
FI-ka371-04	2004	Archipelago Sea	Herring	VHSV (II)	HQ112235	III
ka380_04-ka382_04	2004	Archipelago Sea	Herring	VHSV (II)	HQ112204-HQ112206	III
FI-ka383-04	2004	Archipelago Sea	Herring	VHSV (II)	HQ112236	III
ka384_04,ka385_04,ka387_04	2004	Archipelago Sea	Herring	VHSV (II)	HQ112207-HQ112209	III
FI-ka388-04	2004	Archipelago Sea	Herring	VHSV (II)	HQ112237	III
ka389_04-ka391_04	2004	Archipelago Sea	Herring	VHSV (II)	HQ112210-HQ112212	III

Isolate code	Year of isolation	Origin	Host species	Virus (genotype)	Accession No	Publication
FI-ka392-04	2004	Archipelago Sea	Herring	VHSV (II)	HQ112238	III
ka393_04-ka395_04	2004	Archipelago Sea	Herring	VHSV (II)	HQ112213-HQ112215	III
FI-ka396-04	2004	Archipelago Sea	Herring	VHSV (II)	HQ112239	III
ka397_04	2004	Archipelago Sea	Herring	VHSV (II)	HQ112216	III
ka414_04	2004	Archipelago Sea	Herring	VHSV (II)	HQ112217	III
FI-ka427-04	2004	Archipelago Sea	Herring	VHSV (II)	HQ112240	III
ka428_04	2004	Archipelago Sea	Herring	VHSV (II)	HQ112218	III
FI-ka436-04	2004	Archipelago Sea	Herring	VHSV (II)	HQ112241	III
ka557_04-ka559_04	2004	Archipelago Sea	Herring	VHSV (II)	HQ112219-HQ112221	III
FI-ka560-04	2004	Archipelago Sea	Herring	VHSV (II)	HQ112243	III
ka561_04-ka563_04	2004	Archipelago Sea	Herring	VHSV (II)	HQ112222-HQ112224	III
FI-ka564-04	2004	Archipelago Sea	Herring	VHSV (II)	HQ112244	III
ka565_04,ka570_04,ka574_04	2004	Archipelago Sea	Herring	VHSV (II)	HQ112225-HQ112227	III
FI-ka575-04	2004	Archipelago Sea	Herring	VHSV (II)	HQ112245	III
ka645_04,ka651_04	2004	Archipelago Sea	Herring	VHSV (II)	HQ112228-HQ112229	III
FI-ka646-04	2004	Archipelago Sea	Herring	VHSV (II)	HQ112246	III
FI-ka664-04	2004	Archipelago Sea	Herring	VHSV (II)	HQ112248	III
FI-ka494-05	2005	Archipelago Sea	Herring	VHSV (II)	HQ112242	III
FI-ka350-06	2006	Bothnian Sea	Herring	VHSV (II)	HQ112233	III
ka655_06,ka662_06,ka664_06	2006	Archipelago Sea	Herring	VHSV (II)	HQ112232, HQ112230, HQ112231	III
FI-ka663-06	2006	Archipelago Sea	Herring	VHSV (II)	HQ112247	III
UK-860/94	1994	Gigha, W Scotland	Turbot	VHSV (III)	AY546628	II
DK-4p168	1997	Skagerrak	Herring	VHSV (III)	AY546582	I,II,III
IR-F13.02.97	1997	Ireland	Turbot	VHSV (III)	AY546620	II,III
MLA98/6 PT11	1998	North Sea	Norway pout	VHSV (III)	AY546632	II,III
NO-2007-50-385	2007	Norway	Rainbow trout	VHSV (III)	EU547740	III
FR-L59X	1987	France	Eel	VHSV (III)	AY546618	I,II,III
US-Makah	1988	Washington, USA	Coho Salmon	VHSV (IV)	U28747	I, II,III
JP-KRRV9822	2000	Japan	Japanese flounder	VHSV (IV)	AB179621	I, II,III
ka706_02_grayling	2002	Finland	Grayling	PRV	KC408701, KF146312	IV

Isolate code	Year of isolation	Origin	Host species	Virus (genotype)	Accession No	Publication
ka177_03_perch	2003	Baltic Sea	Perch	PRV	KC408697, KF146314	IV
ka672_03_grayling	2003	Finland	Grayling	PRV	KC408700, KF146311	IV
ka636_09_grayling	2009	Finland	Grayling	PRV	KC408699, KF146310	IV
ka521_09_grayling	2009	Finland	Grayling	PRV	KC408698, KF146309	IV
ka512_09_grayling	2009	Finland	Grayling	PRV	KF146308	IV
ka848_09_sea_trout	2009	Finland	Sea trout	PRV	KF146313	IV
ka501_10_grayling	2010	Finland	Grayling	PRV	KF146315	IV
PRV	1980	France	Perch	PRV	JX679246	IV
grayling_47_ 90	1990	France	Grayling	PRV	-	IV
4890	1990	France	Pikeperch	PRV	JF502607	IV
DK5533	1993	Danemark	Pike	PRV	-, JF502603	IV
N4925	2003	France	Perch	PRV	JF502604	IV
P8350	2004	France	Perch	PRV	JF502609	IV
27V10	2010	Italy	Perch	PRV	JF502605	IV
ka 907_87_brown_trout	1987	Finland	Brown trout	STRV	AF434991	IV
SSTV_28/97	1997	Sweden	Sea trout	STRV	AF434992	IV
O403467	2005	France	Black bass	STRV	JF502596	IV
R6146	2005	France	Perch	STRV	JF502608	IV
EVEX	1986	Netherlands	Eel	AngRV	FN557213	IV
SCRV	?	China	Mandarin fish	SCRV	DQ399789	IV
PFRV	1973	Netherlands	Pike	PFRV	FJ872827	IV
SVCV_Fijan	1971	Yugoslavia	Common carp	SVCV	AJ318079	IV
SVCV_A1	?	China	Common carp	SVCV	DQ097384	IV

* Presumably imported with fish from Denmark
** FiA01.00 is the same as FI-ka-66

4.5 HISTOLOGY (I, IV)

Tissue samples of liver, anterior and posterior kidney, heart and brain or whole grayling fry were fixed in 10% formalin, embedded in paraffin, sectioned at 4 µm and stained with haematoxylin and eosin (H&E) (I, IV). In case of the bigger fry, only the anterior part of the body was treated in the similar way (IV).

4.6 CHALLENGE EXPERIMENT (I, II)

Experimental viral challenge of trout with various VHSV isolates was performed at the Technical University Denmark, National Veterinary Institute, Århus, Denmark.

The challenge trials were designed and performed according to the Danish legislation on the use of animals in experimental trials, under a licence (J.nr 1998/561-71 af 13.oktober 2000) given by "Forsøgsdyrstilsyn" (the Danish Ministry of Justice for Animal Experiments Inspectorate). Care was taken to minimise stressful conditions.

VHSV isolates (listed in Table 7) used in the challenge experiments were of a low (usually 3–7) passage number and were propagated and titrated on BF-2 cells, according to standard procedures (Lorenzen et al. 1988).

Table 7. List of isolates used in the challenge experiment for intraperitoneal (IP) or bath challenge.

Isolate	Virus	Host species	Origin	Year	Publication
FI-ka-66	VHSV genotype Id	rainbow trout	Åland, Finland	2000	I
FiP02a.00	VHSV genotype Id	rainbow trout	Åland, Finland	2000	I
FiP02b.00	VHSV genotype Id	rainbow trout	Åland, Finland	2000	I
FI-672-03	Perch rhabdovirus	grayling	Lake area of Finland	2003	unpublished
FI-lamprey-743-03	VHSV genotype II	lamprey	Finland, Lestijoki	2003	II
DK-3592B	VHSV genotype Ia	rainbow trout	Denmark	1986	I, II
DK-1p53	VHSV genotype II	herring	Baltic Sea	1996	II

The challenge experiments were performed on fingerlings with a mean size of 1.6 g (I) or 7.1 g (II) in a biosecure challenge facility. In publication I, each group was tested in duplicate with 35 to 40 fish in each tank. In publication II, each challenge group consisted of 40 fish divided between two 8-L aquaria, except for the bath challenge group for DK-3592B, which consisted of 60 fish divided between three aquaria. The water temperature was maintained at 9.5–11 °C (I) or 9–12 °C (II, IV). The water used was tap water (not chlorinated). In bath challenge, the water flow was turned off for two hours, and 10 ml (10^5 $TCID_{50}$/ml) (I) or 4 ml (2 x 10^6 $TCID_{50}$/ml) (II) of the challenge or cell culture medium (negative control) was added to each aquarium. For intraperitoneal challenge, fish were anaesthetized

by immersion in 0.01% benzocaine and injected intraperitoneally (IP) with 50 µl virus isolates. During the follow-up period of 21 (I) or 28 (II) days, dead fish were collected daily and examined for clinical signs of VHS. Virological examination was performed on dead fish from each aquarium, either dead post-challenge, or killed by a benzocaine overdose at the end of the trial.

4.7 STATISTICS (I, III)

Cumulative mortalities at the end of the trial were compared within and between the different groups in each experiment by a chi-squared (χ^2) test (Dunn 1977). A P-value < 0.05 was considered to indicate a statistically significant difference. (I)

The prevalence of VHSV infection was analysed with the χ^2-test using PASW Statistix 18 (SPSS Inc., Chicago, Illinois, USA). Bonferroni correction was used in the comparisons of the results of different sampling years (Sokal & Rohlf 1995). (III)

5 RESULTS

5.1 DETECTION OF VIRUS

5.1.1 VHSV (I, II, III)

VHSV genotype Id was isolated from 24 farms in Åland and Uusikaupunki in Baltic Sea and in Pyhtää in the Gulf of Finland during 2000–2004 (I). By the end of 2012, VHSV genotype Id had been isolated 62 times, mainly in Åland in Finland. In some fish farms, VHSV was isolated several times. VHSV genotype II was isolated from 5 pooled lamprey samples originating from the mouth of River Lestijoki (4 samples) and the mouth of the River Kalajoki (1 sample) (II), and from 51 of 758 pooled herring samples from 6 sampling sites in the Archipelago Sea and one sampling site in the Bothnian Sea (III) (Fig. 6 and Table 8). All isolated viruses were identified as VHSV by ELISA and RT-PCR and also by IFAT in publications I and II. The samples tested negative for IHNV, IPNV and SVCV. The genotype Id strains multiplied well on both BF-2 and EPC cells (I); in contrast, EPC cells were not susceptible to infection by genotype II isolates (II, III).

Table 8. Number of farms and sampling sites of wild fish from which VHSV Id and VHSV II were isolated in 2000–2012 in Finland.

	Genotype Id			**Genotype II**				
	Rainbow trout, ***Oncorhynchus mykiss*** (Walbaum)			European river lamprey, ***Lampetra fluviatilis*** (L.)		Baltic herring, ***Clupea harengus membras*** (L.)		
	Åland	**Pyhtää**	**Uusi-kaupunki**	**Mouth of River Kalajoki**	**Mouth of River Lestijoki**	**Archipelago Sea**		**Bothnian Sea**
Year						**• coastal**	**• open**	
2000	1	3						
2001	4	2						
2002	14							
2003	2		1	1	1			
2004	2					3	1	
2005	7					1		
2006	10					1		1
2007	2							
2008	3		1					
2009	6							
2010	1							
2011	3							
2012	1							
Total	**55**	**5**	**2**	**1**	**1**	**5**	**1**	**1**

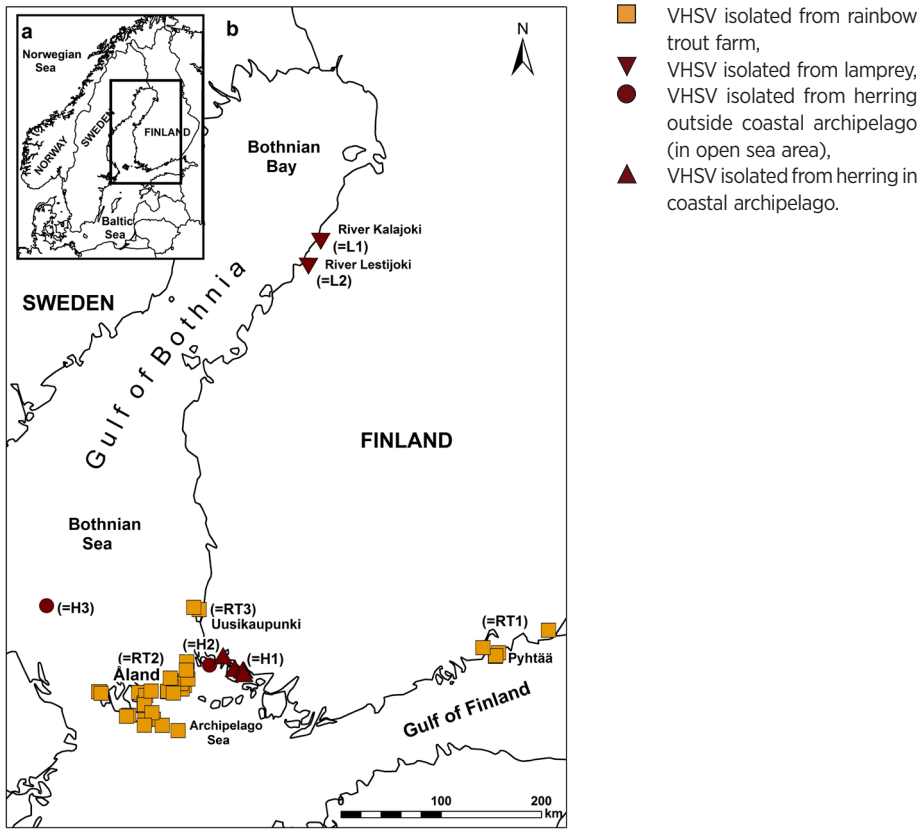

Figure 6. Geographical location of the VHSV strains isolated in Finland.
(a) Orientation map of the study area; the rectangle shows the area of map b.
(b) Location of the VHSV genotype Id strains from rainbow trout (RT1: Pyhtää, RT2: Åland, RT3: Uusikaupunki), genotype II strains from lamprey (L1: mouth of River Kalajoki, L2: mouth of River Lestijoki) and herring (H1: coastal Archipelago, H2: outside coastal Archipelago, H3: Bothnian Sea).

5.1.2 PERCH RHABDOVIRUS (IV)

Perch rhabdovirus was isolated from 12 pooled samples from one farm during 2002, 2009 and 2010 and from one pooled sample from a second farm in 2003. Isolates were initially identified as rhabdovirus based on the typical CPE on BF-2 and EPC cell lines. ELISA tests were negative for the presence of IHNV, IPNV, SVCV and VHSV with all samples studied. BF-2 cells infected with viruses from grayling were stained in IF, applying rabbit antisera against brown trout rhabdovirus ka903_87 and perch rhabdovirus F28. The intensity of the reaction varied from weak to strong. A cross-reaction between brown trout rhabdovirus ka903_87 and perch rhabdovirus was also observed. The most intense staining was observed with both rabbit antisera against some grayling isolates. Ka903_87 isolated from brown trout

exhibited a strong signal with homologous antiserum, but was only weakly stained with the antiserum to perch rhabdovirus F28. VHSV was not detected with IFAT.

Primers GF and GR produced a PCR product of the expected size from brown trout rhabdovirus ka903_87, while no products were obtained from the grayling isolates and perch rhabdovirus ka177_03. The complete G gene sequence was obtained in two overlapping fragments from the grayling isolates and the perch rhabdovirus using primer pairs oPVP116 & oPVP118 and oPVP126 & Rha-G-seqR2, and the length of the open reading frame was 1560 bp for all isolates. Primers LF and LR produced PCR products of the expected size from the grayling isolates and perch rhabdovirus ka177_03, while no products were obtained from brown trout rhabdovirus ka903_87.

5.2 GENETIC ANALYSIS

5.2.1 VHSV (I, II, III)

In publication I, the sequences of the G (1524 nucleotides) and the NV (369 nucleotides) genes and the region between the genes (74 nucleotides) were determined for all 34 Finnish VHS strains isolated between 2000 and 2004 from rainbow trout farms. All isolates were closely related, with 99.3% to 100% nucleotide identity (data not shown). All these isolates were grouped together with a bootstrap value of 96% into genotype I. They formed a sublineage (Id) with a strain isolated in 1968 from rainbow trout in Norway (NO-A163.68, supposedly imported from Denmark) (Einer-Jensen et al. 2004). The first VHSV strain (DK-F1) isolated from rainbow trout in 1962 had 97.8% to 98.4% identity and the DK-3592B isolate used as a positive control in infection trials had 97.2% to 97.5% identity in the G gene with the Finnish rainbow trout farm isolates.

In publications II and III, sequences of the entire G gene were determined for a VHSV strain isolated from wild lampreys from the River Kalajoki (FI-lamprey-739.03), for one strain isolated from the River Lestijoki (FI-lamprey-743.03) and 16 representative isolates from herring. From other lamprey and herring isolates, only partial G gene sequences were determined. Pairwise comparisons of the sequenced regions revealed that all isolates were 99–100% identical (data not shown). All lamprey and herring isolates were grouped together into genotype II with a bootstrap value of 99–100%. They formed a distinct group (genotype II) with strains isolated in 1996 from herring (DK-1p53 and 55) and sprat (DK-1p52) in Gotland. The genetic analysis revealed that the overall difference between strains in the genotype II group was very small and nucleotide homology varied in the range of 99 to 100%. The genotype Ib VHSV isolates from the geographically closest areas, represented by a Swedish marine isolate (SE-SVA-1033) and the Finnish rainbow trout genotype Id isolates shared approximately 89–90% identity with the genotype

II genomes. The genetic relationships of all Finnish VHSV isolates are illustrated in Figure 7, which was constructed from the alignment of the whole G gene.

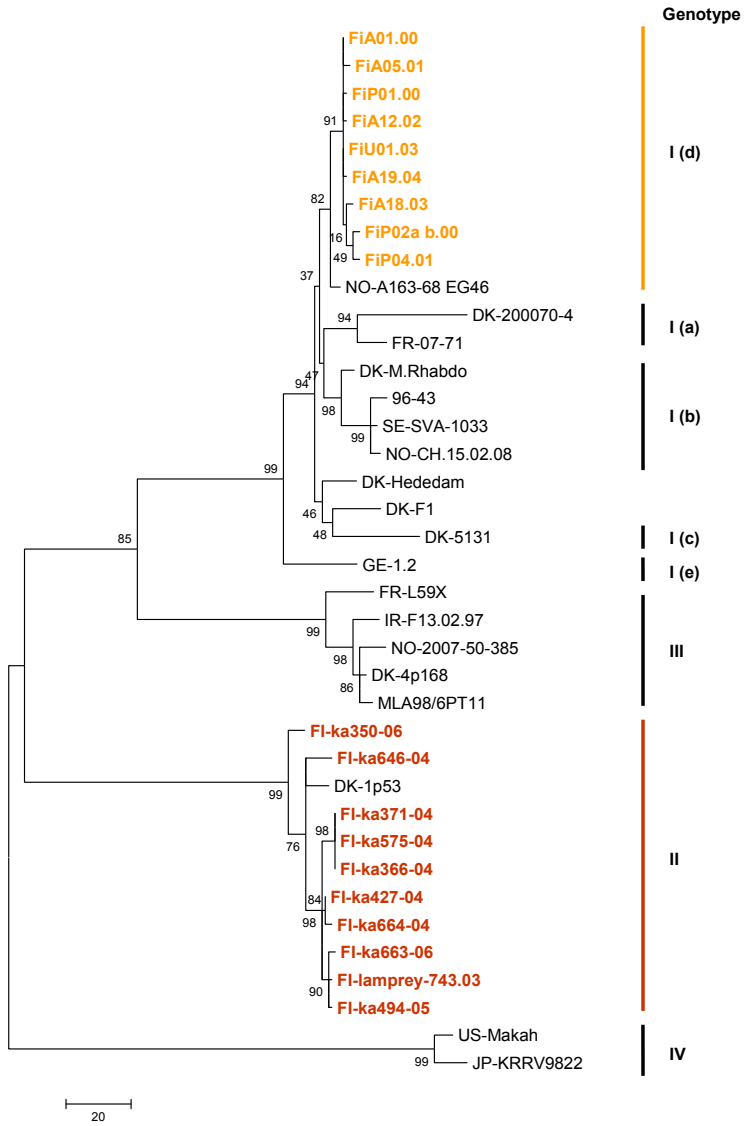

Figure 7. Maximum Parsimony phylogenetic tree obtained using the entire G gene of VHSV. The scale bars indicate the phylogenetic distances. Bootstrap values are indicated at the nodes for a total of 1000 replicates. Sequence alignments were performed using ClustalW2. Phylogenetic trees were constructed using the Maximum Parsimony DNA distance method within the MEGA program. The origin of the representative VHSV isolates used for phylogenetic analysis is shown in Table 6. Finnish isolate names coded as follows: FI or Fi = Finland, A = Åland Islands, P = Pyhtää, U = Uusikaupunki, ka = herring.

5.2.2 PERCH RHABDOVIRUS (IV)

Sequences of the entire G gene (1560 nt) were determined for the six representative grayling isolates and the isolates from wild perch ka 177_03 and sea trout ka848_09 isolated from Baltic Sea, and partial L gene sequences (397 nt) for four representative isolates of grayling strains isolated between 2002 and 2009 and the isolate from wild perch from the Baltic Sea. Pairwise comparisons of full-length G gene and the partial L gene sequences and the phylogenetic analysis revealed that all grayling isolates were closely related to each other, with approximately 98.8–100% nucleotide homology. The Finnish perch isolate ka177_03 was closely related to the grayling isolates, with approximately 95.4% (G gene) and 97.5% (partial L gene) nucleotide homology, while the sea trout isolate ka848_09 had approximately 96.1% (G gene) nucleotide homology (data not shown).

The genetic relationships of representative Finnish perch rhabdovirus isolates are illustrated in Figure 8, which was created from the alignment of the entire G gene. Maximum likelihood analysis of the entire G gene sequences revealed that all Finnish grayling isolates and both the perch and sea trout isolates were most closely related to a perch rhabdovirus isolate from French perch (P8350). They shared 95.1–96.8% identity with Finnish grayling, perch and sea trout isolates. The isolates from perch (PRV, 27V10) and pikeperch (4890) shared 88.4–90.0% identity and the isolate DK5533 from pike shared 88.3–89.1% identity with Finnish grayling, perch and sea trout isolates. Maximum likelihood analysis of partial L gene sequences revealed that all Finnish grayling and perch isolates were most closely related to the isolate DK5533 from pike, sharing 92.2–93.2% identity, and to the first French PRV isolate from perch, sharing 90.7–91.2% identity. Genetic analysis of entire G gene and partial L gene sequences revealed that the Finnish brown trout isolate ka903_87 shared only 66.9–67.2% and 77.6–78.6% identity, respectively, with the Finnish grayling isolates.

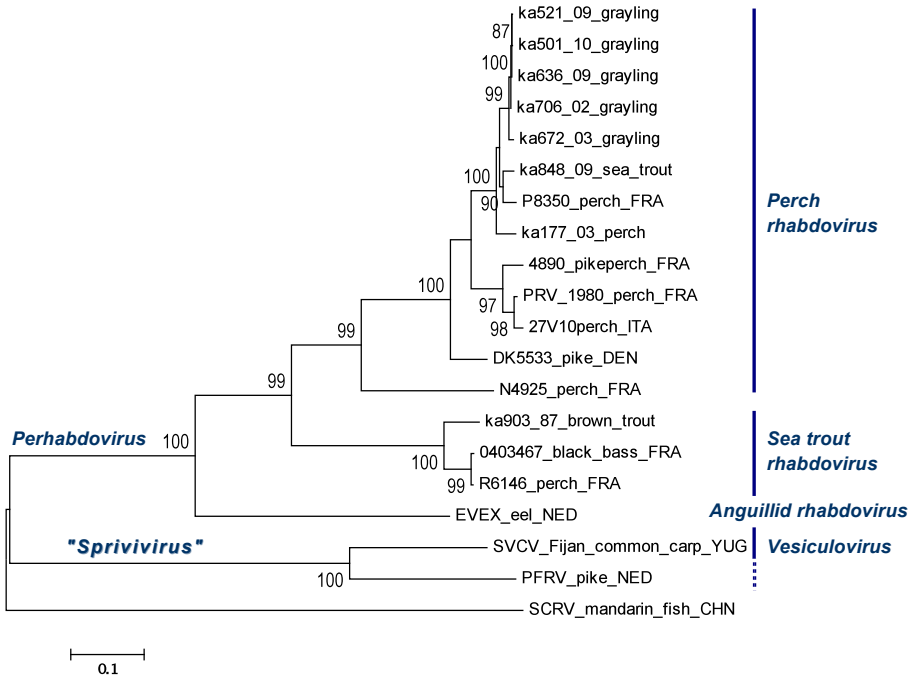

Figure 8. Maximum likelihood phylogenetic tree obtained using the entire G gene of perhabdo-, vesiculo- or vesiculo-type rhabdoviruses. The scale bar indicates nucleotide substitutions per site. Bootstrap values are indicated at the nodes for a total of 1000 replicates and values > 70 are shown on the tree. Sequence alignments were performed using ClustalW2. The phylogenetic tree was constructed using the maximum likelihood method within the MEGA 5 program. The origin of the representative virus isolates used for phylogenetic analysis is shown in Table 6.

5.3 PATHOLOGICAL FINDINGS (I, IV)

In the diseased rainbow trout typical VHS lesions such as anaemia, exophthalmia, epidermal haemorrhages and petechial haemorrhages in the peritoneum, dorsal musculature, heart, gills, swim bladder and visceral adipose tissue were observed in the post-mortem examination. The spleen was clearly enlarged; the kidney was slightly swollen and hyperaemic and in some fish the liver was mottled with hyperaemic areas (I). In histological sections, severe multifocal liver necrosis and prominent necrosis of the tubular cells of the kidney were seen in fish infected with VHSV (I).

For PRV several diseased grayling had a distended abdomen and one grayling had a few haemorrhagic spots in the pyloric area of the abdominal fat, combined with slimy intestinal contents, anaemic inner organs and a patchy liver (IV). Histology revealed peritonitis as an infection of abdominal fat tissue around the pyloric caeca,

and massive necrotic pancreatitis and peritonitis were seen in grayling fry infected with perch rhabdovirus (IV).

5.4 CHALLENGE/INFECTION TRIALS (I, II)

In the case of the Finnish VHSV genotype Id, isolates from rainbow trout produced a cumulative mortality of >81% in the IP injected fish and 38–39% in bath challenged fish when using rainbow trout fingerlings with a mean size of 1.6 g (I). Altogether, 66% of fish died post-IP challenge, whereas only 13% died after the bath challenge when using rainbow trout fingerlings with a mean size of 7.1 g (II). However, 98–100% of fish exposed to the genotype Ia isolate DK-3592B died both in the bath and IP challenge (I, II).

No mortality was observed in the groups of rainbow trout challenged either with the marine FI-lamprey 743.03 or the DK-1p53 genotype II isolates during the trial period (II). The mortality curves (II) are illustrated in Figure 9; the perch rhabdovirus isolated from grayling FI _ka672_03 is also included (unpublished data with the permission of K. Einer-Jensen). No mortality was observed in the groups of rainbow trout challenged with the perch rhabdovirus isolate FI _ka672_03 during the trial period.

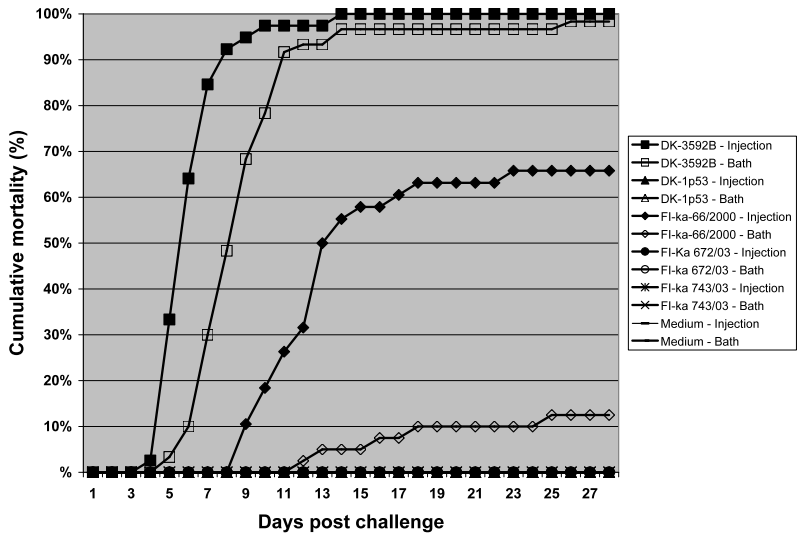

Figure 9. Development of mortality in rainbow trout fingerlings with a mean size of 7.1g after being challenged (IP injection or bath) with the various VHSV isolate and one perch rhabdovirus isolate from grayling, FI-ka672_03. Each curve represents the cumulative percentage mortality in 2 aquaria, each with 20 fish. The only exception was the bath challenge with isolate DK-3592B, which was carried out using 3 aquaria (60 fish in total) (II).

5.5 ASSOCIATION OF VHSV INFECTION IN BALTIC HERRING WITH EPIDEMIOLOGICAL BACKGROUND FACTORS (III)

The frequency of positive herring pools was highest (12%) in the Archipelago Sea of the southwestern coast of Finland, 0.3% in the Bothnian Sea to the west of Finland, and no positive fish were detected in the Eastern Gulf of Finland of the southeast coast of Finland. The relationship between the sea area and VHSV isolation was highly significant (χ^2 test, $P < 0.001$). In total, 556 samples were collected during May–June 2004–2006, of which 51 were positive, while 202 samples collected during November to April were all negative. The majority of the isolations (46, ca. 16% of all samples from that ecosystem) were from the coastal archipelago. The corresponding value for the pooled samples from the open sea was 5 (ca. 1%; χ^2 test, $P < 0.001$). The distribution of positive samples between the years 2004–2006 was uneven: 16% of the pooled samples were positive in 2004, but only 0.4% in 2005 and 2% in 2006 (Bonferroni adjusted χ^2 test, $P < 0.01$ was significant between 2004 and 2005 or 2006, but no significant difference was found in the rate of VHSV-positive isolations between 2005 and 2006).

6 DISCUSSION

6.1 GENETIC DIVERSITY WITHIN FINNISH VHSV ISOLATES (I, II, III)

The Finnish rainbow trout VHSV isolates are grouped based on the analysis of the G gene in genotype Id with an old Norwegian isolate (NO-A163-68EG46), supposedly imported from Denmark (Einer-Jensen et al. 2004). Genotype Ib has been isolated from wild fish from the Baltic Sea, but also from a marine cultured rainbow trout farm in Sweden, from the Kattegat and from the North Sea (Nordblom 1998; Nordblom & Norell 2000). Danish freshwater rainbow trout isolates mainly cluster in genotype Ia, but some isolates from earlier dates cluster in genotype Ic (Kahns et al. 2012). Finnish rainbow trout genotype Id isolates have been shown to be genetically more closely related to the old freshwater isolates and to the first marine isolate from Denmark than to the recent isolates, and thus to be located close to the ancestral source in the phylogenetic trees (Einer-Jensen et al. 2004). The closest relative was found to be the marine isolate DK-M.Rhabdo, which was isolated from cod (identity 98.4–98.9%). The first VHSV strain, DK-F1, is also serologically and genetically closely related to the Finnish VHSV genotype Id strain. Phylogenetic analyses have indicated that the European genotype I lineages have a common ancestral source (Einer-Jensen et al. 2004).

Finnish VHSV genotype Id isolates are closely related genetically, with only 3–4 bases differing at the beginning of the G gene in three of the isolates. This difference did not affect virus pathogenicity in experimental infection, as one of the Finnish isolates used in infection experiments had this change. The previously reported variable region V2 in the G gene, where many amino-acid substitutions have been reported to accumulate (Benmansour et al. 1997), showed 100% amino-acid homology in our genotype Id isolates. The non-coding region between the G and NV genes showed more variation than the coding regions between the isolates and was useful while examining similar virus strains. 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 appears to be more useful for genetic analysis among closely related strains.

According to Einer-Jensen et al. (2004), molecular clock analyses of the VHSV G gene sequences indicated that rates of substitution were not equal throughout the phylogenetic tree, but that a molecular clock hypothesis could be accepted for two subsets of the data representing either trout farm genotype Ia isolates or marine genotype isolates Ib, II, III and IV. For these groups, the likelihood ratio test supported the hypothesis of molecular clocks with substitution rates of 1.74

x 10^{-3} and 7.06 x 10^{-4} substitution per codon per year, respectively (Einer-Jensen et al. 2004). The mutation rate of the Finnish rainbow trout VHSV genotype Id was found to be low, since the isolates collected at different times / years from the same fish farms were almost all identical. Assuming a common ancestor for the Finnish isolates and the Norwegian genotype Id isolate from 1968, the estimated nucleotide substitution rate is 1.3 x 10^{-4} to 2.6 x 10^{-4} / site / year. This is within the range of the lowest evolutionary rates estimated for other RNA viruses (Jenkins et al. 2002), but lower than was previously reported for other VHSV isolates (Einer-Jensen et al. 2004).

Before VHSV genotype II isolates from lamprey caught in the mouths of the rivers Lestijoki and Kalajoki and herring from the Archipelago Sea were identified, genotype II only included isolates from fish caught in the narrow region referred to as the Eastern Gotland Basin (Baltic Sea) (Einer-Jensen et al. 2004). All herring and lamprey isolates were grouped together into genotype II with a bootstrap value of 100%, forming a distinct group (genotype II) with strains isolated in 1996 from herring and sprat in Gotland. The genetic difference between strains in the genotype II group was very small and varied in the range of 99–100%. Genotype II appears to represent an additional marine lineage, with no clear link to rainbow trout aquaculture (Snow et al. 2004). According to our study, genotype II appears to be more widespread than previously known. At present, it is the only genotype in wild fish around the Finnish coast of the Baltic Sea. Genotype Ib has been isolated from various species in the southern parts of Baltic Sea, especially around Bornholm (Skall et al. 2005b). The presence of two relatively different genotypes within the Baltic Sea could be a result of divergent evolution within different biotypes and/or hosts.

6.2 VIRULENCE OF FINNISH VHSV ISOLATES (I, II, III)

VHSV isolates from wild marine fish are serologically (Einer-Jensen et al. 1995; Benmansour et al. 1997) and genetically (Einer-Jensen et al. 2004; Snow et al. 2004; Campbell et al. 2009) similar to those from fish farms, but marine isolates typically produce little or no mortality in rainbow trout fry following waterborne challenge (Dixon et al. 1997; Campbell et al. 2009). It has been experimentally shown that the growth of a non-pathogenic marine isolate is severely inhibited at the initial stage of virus infection in immersion-challenged rainbow trout (Campbell et al. 2011). Differences within the G protein have been shown to affect the pathogenicity of novirhabdoviruses (Benmansour et al. 1997; Gaudin et al. 1999; Betts & Stone 2000; Campbell et al. 2009; Cho et al. 2012). According to Campbell et al. (2011), the low virulence of a marine isolate of VHSV in rainbow trout was more related to defects in the initial entry and/or replication of the virus than defects in the suppression of the host IFN response. NV protein of VHSV may play an important

role not only in viral replication but also in pathogenesis (Kim et al. 2011). NV has a role in delaying apoptosis and limiting the host IFN response in fish (Ammayappan & Vakharia 2011; Choi et al. 2011). The NV gene was highly conserved, suggesting its essential role. The virulence factors influencing the growth of genetically similar VHSV isolates have not yet been determined (Cho et al. 2012).

In study I, rainbow trout fingerlings of 1.6 g in size were used. The Finnish rainbow trout VHSV genotype Id (FiA01.00) induced a mortality of approximately 40% when infected by immersion, which is lower than the mortality in infection trials using VHSV isolates from recent outbreaks in freshwater rainbow trout farms (Skall et al. 2004). After intraperitoneal injection, the Finnish genotype Id isolates were highly pathogenic, causing mortality of approximately 90%. In study II, rainbow trout fingerlings of 7.1 g in size were used. In this case, the injection of Finnish rainbow trout VHSV genotype Id (FiA01.00) resulted in 66% mortality, whereas only 13% died after the bath challenge. Previous experimental infection studies in rainbow trout have revealed moderate to high mortality, ranging from 30–90% depending on the fish size, inoculation doses and temperatures, induced by the VHSV reference strain DK-F1, which is serologically and genetically closely related to the Finnish VHSV genotype Id strain (Jørgensen 1970; Jørgensen 1974). On the other hand, VHSV strains isolated from wild populations of marine fish have induced no or less than 5% mortality in rainbow trout when infected by immersion (Skall et al. 2004). The marine DK-M.Rhabdo is genetically very closely related to Finnish VHSV genotype Id (98.4–98.9% identity); however, it did not cause any mortality by immersion (Skall et al. 2004). Finnish rainbow trout genotype Id isolates could represent an intermediate stage of marine strains evolving towards pathogenicity in rainbow trout. When infected by intraperitoneal injection, however, the Finnish isolates were highly pathogenic. The infected fish showed signs typical of VHS, and VHSV was isolated from the fish that died and also from most of the surviving fish (examined in pools of 1–10 fish) in tanks with fish infected by immersion and IP.

No mortality was observed in the groups of rainbow trout challenged either with the marine FI-lamprey 743.03 or the DK-1p53 genotype II isolates. However, 98–100% of fish exposed to the genotype Ib isolate DK-3592B from freshwater-reared rainbow trout died both in study I and II. According to experimental infections, European VHSV genotypes show different pathogenicity patterns depending on the host species, although the phylogeny does not directly reflect the host range. European VHSV isolates originating from freshwater reared rainbow trout often belong to genotypes Ia, Ic, Id or Ie and are generally highly pathogenic to rainbow trout, but show no or low pathogenicity to marine fish species. However, isolates originating from marine fish species frequently belong to genotypes Ib, II or III and often show no or very low pathogenicity to rainbow trout (Skall et al. 2004), dividing the European VHSV isolates into rainbow trout-adapted or non-adapted isolates, respectively (Schönherz et al. 2012b). Dale et al. (2009) diagnosed pathogenic VHSV

genotype III isolates for the first time in a Norwegian rainbow trout farm. A non-pathogenic form of VHSV may become more virulent after passage through rainbow trout (Snow & Cunningham 2000; Skall et al. 2004). VHSV in wild marine fish can represent a potential threat to rainbow trout cage farms located in the marine environment. New virulent strains may evolve from genotypes that are non-virulent at present, as they quite likely have done in the past (Nordblom 1998; Nordblom & Norell 2000; Dale et al. 2009).

6.3 EPIDEMIOLOGY OF FINNISH VHSV ISOLATES (I, II, III)

VHSV genotype Id was first isolated in 2000 in rainbow trout farms both in Åland in the southwestern Finnish Archipelago Sea and Pyhtää on the coast of the Gulf of Finland. The farms were situated 330 km apart and they had no connection between each other. The outbreaks occurred almost simultaneously in late April and May. The origin of the first outbreaks is still unclear. European VHSV isolates that are adapted to rainbow trout and are highly pathogenic to this species have been assigned to genotypes I and III (Snow et al. 1999; Einer-Jensen et al. 2004; Dale et al. 2009). According to Einer-Jensen et al. (2004), phylogenetic studies have revealed that rainbow trout isolates most likely evolved from a marine ancestor that managed to cross species barriers. Our genotype Id outbreaks in rainbow trout farms and the outbreaks in marine cultured rainbow trout in Sweden (Nordblom 1998; Nordblom & Norell 2000) and Norway (Dale et al. 2009) support this hypothesis. These isolates were genetically more closely related to isolates from marine species from the surrounding waters than to isolates from freshwater cultured rainbow trout (Einer-Jensen et al. 2004; Dale et al. 2009), indicating that inter-species transmission from marine to cultured species occasionally occurs (Kurath & Winton 2011).

A freshwater origin from inside Finland seems unlikely, because the mainland is free of VHSV, and farms supplying fingerlings were shown to be free of the virus. 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. Without the long temporal distance, the short genetic distance to the old Danish isolates would suggest the original source of the infection to be there. Assuming the infections to originate from wild fish populations, e.g. herring, it is natural that some genetic variation should occur, but genotype II was isolated from both lamprey and herring. Finnish rainbow trout genotype Id and the genotype II isolates are not closely related (approximately 89–90% identity).

The ancestral source of VHSV could still exist somewhere in the Baltic Sea. There is a close genetic linkage between VHSV in the marine environment and VHSV in farmed rainbow trout in Europe, and adaptation of the virus to rainbow

trout is suggested to be a relatively recent event that has occurred more than once during the past 50 years in European rainbow trout farming (Einer-Jensen et al. 2004). The epidemic of VHS in Finnish rainbow trout farming could also be due to a recent re-introduction from an unknown source. Finnish food fish production of rainbow trout mainly involves marine production systems. In comparison with tank or pond farming, the interaction between rainbow trout and wild fish is much greater in marine production systems, where trout are typically held in net cages, sharing their environment with wild fish, which are potentially attracted by feeding activities. Net cages also allow the invasion of small wild fish. Some wild fish populations that circulate close to Finnish fish farms could be VHSV carriers. The existence of marine VHSV reservoirs and the high evolutionary potential of RNA viruses in general represent potential risk factors for host shifts from marine fish to rainbow trout (Schönherz et al. 2012a). Transmission of VHSV is mainly established by the horizontal transport of viral particles through the water (Kurath & Winton 2011), by direct contact with infected fish, or by oral ingestion of infected material (Schönherz et al. 2012a). Accordingly, infected wild fish are suspected to represent a threat to semi-closed aquaculture systems, such as sea-cage systems of marine cultured rainbow trout. In these systems, cultured and wild fish share the same environment (Schönherz et al. 2012b). The original introduction of VHSV to cultured rainbow trout may have occurred through oral transmission because of feeding of unpasteurized marine fish products, which was previously a common practise (Meyers & Winton 1995; Skall et al. 2005a; Campbell et al. 2009; Snow et al. 2009). Snow et al. (2009) did not demonstrate oral transmission in Atlantic cod when fish were fed with VHSV-infected fish pellets. According to Schönherz et al. (2012a), an oral transmission route of VHSV in rainbow trout exists, and rainbow trout can be infected with VHSV by ingesting infected material. Schönherz et al. (2012a) demonstrated that VHSV is able to replicate in the fish stomach as well as kidney tissue, and that stomach tissue is a site of primary viral replication following oral exposure to VHSV. Infection through oral transmission progresses slower than infection through waterborne transmission, however (Schönherz et al. 2012a). One reason for the Finnish outbreaks could be the feeding of fish with raw wild fish, as was previously done in Finnish fish farming. Infected farms in Pyhtää used acid-treated Baltic herring and slaughter waste in their semi-moist fish feed. The first VHSV-positive farm in Åland used only commercial dry fish feed, but it was situated close to a fishing harbour. Transmission of VHSV to rainbow trout, however, requires that the virus can replicate to a sufficient extent in both marine fish species and rainbow trout (Schönherz et al. 2012b). Human activities are most certainly very important in the rapid spread of the virus in rainbow trout farming after the index case.

The spread of VHSV in the marine environment could occur via predation on infected herring, because many marine species feed on herring (Meyers et al. 1994,

Meyers & Winton 1995; Meyers et al. 1999). Herring and sprat are the main prey fish of Baltic salmon (Karlsson et al. 1999). Our negative results from the salmonid broodfish material, however, do not support the importance of the peroral infection route in grayling, whitefish, sea trout or Baltic salmon. In European marine waters, where different genotypes of the virus exist, there may be different host sources of the virus, or different populations of virus in herring stocks (Dixon et al. 1997). Lampreys are potential candidates for mechanical transfer of VHSV because of their parasitic feeding habits on live fish. Moreover, the pH in their gastro-intestinal tract is not suitable for the inactivation of VHSV and they are able to move from host to host (Hardisty 1979; Neukirch 1985). Viral concentrations in the gastro-intestinal tract could reach higher loads than in the surrounding open water body, so in naïve populations, oral transmission might be a mechanism assisting host adaptation (Schönherz et al. 2012a). Lampreys attach themselves to herring or sprat used as bait on salmon drift lines, and scars have been found on herring and cod along the Finnish coast (Tuunainen et al. 1980), but have not been reported on salmon, sea trout or whitefish. According to a local fish farmer (Väätäjä Juha, local fish farmer, pers. comm.), lamprey scars or attacks on farmed rainbow trout have not been observed in the farms near rivers of the Bothnian Bay where lampreys migrate. The close proximity of fish in schools may also enhance the transmission of the virus via water and fish-to-fish contact. In marine aquaculture of rainbow trout, preying on invading wild fish might thus be a risk factor for the introduction and adaptation of VHSV and subsequent disease outbreaks (Schönherz et al. 2012a).

Further estimation of the prevalence of infection in the rivers Lestijoki and Kalajoki proved to be difficult, because the sample sizes were limited. In our study, VHSV genotype II was detected from 51 out of 758 pooled herring samples when a total of 7580 herring were examined during 2004–2006. All positive samples were caught during May–June, the main spawning period of Baltic herring. The majority of the isolations were from the coastal archipelago, where the herring has its spawning grounds. The distribution of our positive samples between the years 2004–2006 was uneven. Predisposing factors such as spawning, hormonal factors, migration, a high population density and low water salinity can explain the high proportion of positive VHSV samples in this study in comparison with the earlier results from the Baltic Sea (Mortensen et al. 1999; Einer-Jensen et al. 2009). That we simply managed to discover a cluster of VHSV-infected herring that were there by chance, especially in 2004, is also a possibility. Skall et al. (2005) occasionally recorded VHSV genotype Ia at a prevalence of 17% among herring around Bornholm in Denmark during April to May, but VHSV was not detected in fish sampled mainly from open sea areas north of Gotland. The prevalence of VHSV is also low in populations of wild adult Atlantic herring, with virus occurring in only 1/3711 from the North Sea and coastal waters of Scotland (King et al. 2001b), in 11/212 pooled samples of 2100 herring from waters near Denmark (Mortensen

et al. 1999) and 0/1937 samples from Scottish waters (Matejusova et al. 2010). VHSV was certainly more prevalent in the Baltic Sea in an area between Zealand and the island of Bornholm and the waters surrounding Bornholm than in the Kattegat, Skagerrak and along the North Sea coast of Denmark (Skall et al. 2005b). According to Lovy et al. (2012), it is likely that a very small percentage of Pacific herring were carrying VHSV when captured. Then, factors associated with their confinement resulted in increased infection pressures, including reduced water exchange rates and an increased probability of contact with shed virions, resulting in an epizootic cascade.

6.4 SUSCEPTIBILITY OF BF-2 AND EPC CELL LINES TO FINNISH RHABDOVIRUS ISOLATES (I, II, III)

The VHSV genotype Id isolates from rainbow trout replicated in both BF-2 and EPC cell lines, whereas VHSV genotype II isolates from herring and lamprey grew only in the BF-2 cell line in this study. For the isolation of VHSV, BF-2 and RTG-2 cells performed equally well and had higher sensitivity than other cell lines (Lorenzen et al. 1999). BF-2 cells were shown to be superior to EPC and CHSE-214 cells for the isolation of VHSV from clinically infected fish and fish without clinical signs by Olesen & Jørgensen (1992). According to Dixon et al. (1997), VHSV isolated from Atlantic herring from the English Channel was not detected in EPC cells. The use of more than one cell line is needed for the most reliable results when attempting to isolate viruses from fish.

6.5 CHARACTERIZATION OF PERCH RHABDOVIRUS ISOLATED FROM GRAYLING (IV)

According to ICTV (2013b), the new *Perhabdovirus* genus has three species: *Perch rhabdovirus* (PRV), *Anguillid Rhabdovirus* and *Sea trout rhabdovirus* (STRV). Siniperca chuatsi rhabdovirus (SCRV) (Tao et al. 2008) isolated from mandarin fish or Chinese perch shares some similarity with perhabdoviruses, but is not considered as a member of the genus *Perhabdovirus*, at least not yet (ICTV 2013b). Talbi et al. (2011) divided similar fish vesiculo and vesiculo-type viruses into two major clusters (I and II). All rhabdoviruses isolated from perch fell into cluster I, which was divided into eight distinct genogroups (A-H) supported by high bootstrap values (76–100%) with G and partial N gene sequences (Talbi et al. 2011). SVCV and PRFV fell into cluster II. Isolates sharing more than 93.7% nucleotide identity (based on partial N and G gene sequences comparison) belong to the same group. Genogroup A contained rhabdoviruses isolated from perch in France and Italy between 1980 and

2002. Genogroup B included perch rhabdoviruses that were isolated from farms in the same region between 2004 and 2009. Genogroups C and D each contained only one rhabdovirus, isolated from pike in Denmark (DK5533) and perch in France respectively. Genogroup E consisted of two nearly identical isolates from perch and black bass from France and an isolate from sea trout from Sweden (SSRV) and from brown trout from Finland (ka903_87). EVEX and SCRV fell into the genogroups G and H, respectively (Talbi et al. 2011).

Phylogenetic analysis of both entire G and partial L genes placed grayling isolates in the perch rhabdovirus within perhabdoviruses in our study. G gene sequences revealed that all Finnish grayling isolates, both the perch and sea trout isolates, were most closely related to perch rhabdovirus isolate from France in 2004 and shared approximately 96.1% identity. Isolates from perch from France in 1980 and from Italy in 2010 and pikeperch from France in 1990 shared approximately 89.5–90.0% identity with our grayling virus. The isolate DK5533 from pike from Denmark in 1994 shared approximately 88.9% identity in analysis of G gene sequences and approximately 92.8% identity in analysis of partial L gene sequences. Partial L gene sequences also revealed that all Finnish grayling and perch isolates were closely related to the French PRV isolated in 1980 from perch, sharing approximately 90.1% identity. The genetic analysis of entire G gene and partial L gene sequences revealed that the brown trout isolate ka907_87 shared only approximately 67.0% and 78.2% identity, respectively, with our grayling isolates.

These results are consistent with a previous analysis of a conserved region of the L gene, in which PRV were clearly separated from SVCV, PFRV and ka903_87 (Betts et al. 2003). However, one of the newly described French isolates from genogroup E - or according to ICTV (2013b), sea trout rhabdovirus - was more distantly related to the others, displaying a relatively high divergence from the other perch viruses (Talbi et al. 2011). Our phylogenetic analysis also confirmed a split between a genetic cluster formed by SVCV and PFRV and a cluster (genus *Perhabdovirus*) containing PRV and a number of grayling, perch, black bass and pikeperch isolates, including those described in this study and by others (Dorson et al. 1984; Nougayrede et al. 1992; Jørgensen et al. 1993; Johansson et al. 2001; Johansson et al. 2002; Talbi et al. 2011; ICTV 2013b).

The brown trout rhabdovirus ka903_87 isolated from brown trout in Finland in 1987 was shown to cross-react with antibodies to a rhabdovirus isolated from asymptomatic pike in the IFAT (Koski et al. 1992; Jørgensen et al. 1993; Björklund et al. 1994), and is also genetically related to the Swedish sea trout virus (SSTV) isolated in 1996 from sea trout from the archipelago of Stockholm, Baltic Sea (Johansson et al. 2001). However, the primers GF and GR designed in this study based on the published glycoprotein gene sequences of brown trout rhabdovirus ka903_87, spring viremia of carp virus and pike fry rhabdovirus did not amplify any of the grayling virus isolates, although PAb ka903_87 cross-reacted with grayling isolates

in IFAT in our laboratory. The first isolation of a rhabdovirus from perch in Norway cross-reacted serologically with brown trout rhabdovirus ka903_87 (Dannevig et al. 2001), but unfortunately no sequence data are available. It appears to be difficult to distinguish between perch rhabdovirus and other perhabdoviruses with the serological assays recommended by OIE (Rowley et al. 2001; Way et al. 2003; Dixon & Longshaw 2005). It was impossible to distinguish between brown trout rhabdovirus ka903_87 and grayling isolates by IFAT in our laboratory.

The Finnish grayling isolates were not pathogenic to rainbow trout fingerlings by immersion or intraperitoneal injection under experimental conditions at 9–12 °C (unpublished data). The Norwegian perch and the Danish pike rhabdovirus were not pathogenic to rainbow trout, either (Jørgensen et al. 1993; Dannevig et al. 2001). There is only limited data on the pathogenicity of the perch rhabdoviruses (Dorson et al. 1984; Nougayrede et al. 1992). Nonetheless, there is some strong evidence to suggest that these viruses pose a serious threat to both cultivated and wild fish populations (Betts et al. 2003). The viruses were associated with high mortality in the fish species from which they originated, and in the absence of other pathological agents were considered to be the primary aetiological agent (Betts et al. 2003). The experimental bath infection study in pike fry revealed a mortality level up to 51% and 87% (Dorson et al. 1987; Jørgensen et al. 1993). Many virus isolations were carried out from healthy asymptomatic fish, but in some species an additional trigger may be required to induce the clinical disease following a natural infection (Rowley et al. 2001; Betts et al. 2003). Perch rhabdoviruses have occasionally exhibited a high level of pathogenicity and may pose a significant threat to both cultivated and wild freshwater fish populations.

The origin or reservoir of the Finnish grayling rhabdovirus has not been clarified. Talbi et al. (2011) observed a very close genetic relationship between one French perch rhabdovirus and Finnish brown trout rhabdovirus ka907_87, although others were closely related to our grayling, perch and sea trout isolates. Additionally, a similar relationship was observed between an isolate from diseased perch from Lake Arungen, which has a connection to seawater in Norway, and the Finnish brown trout rhabdovirus ka907_87 (Dannevig et al. 2001). Brown trout may carry a PFRV-related virus under farmed conditions without symptoms (Adair 1986; Rowley et al. 2001). Migrating trout and pike might be the reservoir of viruses that infect perch and other freshwater species such as grayling (Talbi et al. 2011). Subclinical vesiculo-type rhabdovirus infection of brown trout has been described since 1986, when it was shown that individuals may carry a PFRV-related virus under farmed conditions without symptoms (Adair 1986; Rowley et al. 2001). Thus, brown trout may have an important role as a carrier host when migrating from the marine environment to freshwater (Talbi et al. 2011). Similarly to Talbi et al. (2011), we also confirmed a rather close genetic relationship between our grayling isolates and PRV and the pike isolate DK5533. However, the Finnish brown trout rhabdovirus ka903_87 isolate

is clearly distinguishable from all other Finnish isolates originating from grayling, perch and sea trout. The possible role of the brown trout rhabdovirus as a threat to grayling aquaculture remains unclear. Our results also indicate that sea trout might have a role in carrying the perch rhabdovirus from the marine environment to freshwater. However, there has been no migration of sea trout or other migratory fish between the Baltic Sea and the regions of the fish farms or the location of the wild brood grayling in this study for thousands of years due to natural migration barriers in the river systems. Thus, other mechanisms than natural fish migration from the sea are probably a more likely source of the virus in this study.

7 CONCLUSIONS

1. VHSV genotype Id was diagnosed for the first time in 2000 from four Finnish rainbow trout farms in brackish water. The infection has spread since then and VHSV genotype Id has been isolated from rainbow trout farms in three separate areas, two in the Baltic Sea and one in the Gulf of Finland. The Finnish VHSV genotype Id induced lower mortalities than freshwater VHSV isolates in infection experiments in rainbow trout. It could represent an intermediate stage of marine isolates evolving towards pathogenicity in rainbow trout.

2. VHSV genotype II was isolated for the first time in European river lamprey samples from the rivers Lestijoki and Kalajoki, which flow into the Bothnian Bay of the Baltic Sea. The virulence of the lamprey genotype II isolate was evaluated by an experimental infection trial in rainbow trout fry. No mortality was induced post-infection by waterborne or intraperitoneal challenge, while two genotype Id isolates originating from Finnish rainbow trout caused marked mortality in rainbow fry under the same conditions.

3. VHSV genotype II was isolated from herring mainly from the coastal archipelago, especially from fish caught during the spawning season. All the isolates were closely related, with 98.8–100% nucleotide identity, which suggests the same origin of infection. They were also closely related to the VHSV strains isolated from European river lamprey.

4. Two different VHSV genotypes are circulating in Finland. The origin of VHSV genotype Id in Finnish rainbow trout farms is still unclear. The high evolutionary potential of RNA viruses and existence of marine VHSV strains are potential risk factors for host shifts from marine fish to rainbow trout. Based on our findings, it seems that the infections of herring and lamprey with VHSV genotype II are not directly linked to the rainbow trout infections in Finland. We cannot rule out the possibility that VHSV genotype II could evolve to become more virulent.

5. Perch rhabdovirus was isolated from diseased farmed grayling fry in Finland. The same virus was also isolated from wild perch and sea trout from the Baltic Sea. All Finnish perch rhabdovirus isolates were closely related. Of the viruses reported from other countries, the closest nucleotide identity was with perch rhabdovirus from France in 2004, and then with perch and

pikeperch isolates from France, a perch isolate from Italy and a pike isolate from Denmark. The origin or reservoir of the Finnish grayling rhabdovirus has not been clarified. Wild fish such as trout might have a role in carrying the perch rhabdovirus from the marine environment to freshwater; however, this is unlikely to be the source of the virus in this study.

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