Viral haemorrhagic septicaemia in Finnish brackish water fish farms

Studies on disease surveillance and epidemiology of viral haemorrhagic septicaemia virus

Pia Vennerström

ACADEMIC DISSERTATION

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Abstract

Viral haemorrhagic septicaemia (VHS) was isolated for the first time in Finland in 2000 from a Finnish brackish water fish farm farming rainbow trout in net pens in the Province of Åland, Baltic Sea. The efforts to eradicate the disease from the Åland islands were not successful. Epidemical factors, needed for VHS management in viral haemorrhagic septicaemia virus (VHSV) positive brackish water fish farms, were studied in a 3-year project, the results of which are presented in this thesis. The study compared the ability of four different surveillance procedures and three diagnostic tests to reveal whether a fish population was infected with VHSV. The programme that was conducted as syndromic surveillance, where the farmers sent in samples for diagnostics if any signs of possible fish disease were noticed, clearly outperformed the other three programmes, which were based on active surveillance. A real-time reverse transcriptase polymerase chain reaction method proved to be at least as sensitive in detecting acute VHSV infections as virus isolation in cell culture, which is considered the gold-standard method for diagnosing VHSV. An ELISA method was used to test fish sera for antibodies against VHSV and was found to be a promising tool in VHSV eradication, particularly for screening populations during the follow-up period, before declaring an area free of infection.

During the epidemics it was a common suspicion wild fish being the most likely source of the reinfections of VHSV in infected fish farms in the restriction area. Wild fish of 17 different species from VHS-positive fish farms were screened for VHSV during 2005-2008. In addition, uninfected wild perch, roach and farmed whitefish were introduced to a fish farm with rainbow trout experiencing a clinical outbreak of VHS. The wild fish did not test positive on any occasion, but whitefish were infected and started to replicate VHSV for a short time. The replication of the virus in whitefish was verified using a new qRT-PCR method that tests separately for positive- and negative-sense viral sequences in infected organ samples.

The presence of VHSV in the environment on fish farms or processing plants farming or handling VHSV-positive fish was studied by testing samples for VHSV from wild blue mussels (*Mytilus edulis*) living in infected fish farms. Sea water and sediment from infected fish farms were also tested for VHSV. Wild uninfected blue mussels were also challenged with VHSV in two different challenge tests. Wastewater from a processing plant was tested before and after disinfection treatment. Blue mussels were not found to be carriers of VHSV on any occasion. Sea water tested positive for VHSV RNA more often during the wintertime when water temperature was close to 0°C and sunlight (UV light) sparse. Most wastewater samples collected before the disinfection treatment were positive for VHSV, but samples collected after disinfection were all negative regarding VHSV RNA. Contacts between the processing plants and the fish farms in the restriction area of VHS were very common during this study. Processing plants are usually the place where fish food and farming equipment are stored, including boats that are used for the daily servicing of the farming localities. According to the results of this study, this contact was considered a major risk for disease spread, especially during the cold part of the year when daylight is also short.

Altogether, this thesis compiles the results of a series of studies targeting factors that could affect the infection pressure of VHSV on disease free fish populations.
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List of original publications

This thesis is based on the following publications:


The publications are referred to in the text by their roman numerals.
Abbreviations

BF-2 Bluegill fry (cell line)
cia. circa (Latin), about
CPE cytopathic effect
Ct threshold cycle cut-off
DNA deoxyribonucleic acid
DTU Technical University of Denmark
EC European Commission
e.g. exempli gratia (Latin), for example
ELISA enzyme-linked immunosorbent assay
EM electron microscopy
EPC Epithelioma papulosum cyprini (cell line)
EURL Community reference laboratory for fish diseases
FI Finland
G glycoprotein
ICTV International Committee on Taxonomy of Viruses
i.e. id est (Latin), that is
IFN interferon
IgG immunoglobulin G
IgM immunoglobulin M
IHN(V) Infectious haematopoietic necrosis (virus)
IPN(V) Infectious pancreatic necrosis (virus)
L RNA polymerase protein
M matrix protein
MAb monoclonal antibody
MEM minimum essential medium
N nucleoprotein
NV non-virion protein
OIE World Organisation for Animal Health (Office International des Epizooties)
<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>P</td>
<td>phosphoprotein</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PNT</td>
<td>plaque neutralisation test</td>
</tr>
<tr>
<td>PPR</td>
<td>pattern recognition receptors</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reversed transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TCID50</td>
<td>50% tissue culture infective dose</td>
</tr>
<tr>
<td>VHS(V)</td>
<td>Viral haemorrhagic septicaemia (virus)</td>
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1 Introduction

Viral haemorrhagic septicaemia is a fish disease that is caused by viral haemorrhagic septicaemia virus (VHSV), a virus belonging to the genus *Novirhabdovirus* of the *Rhabdoviridae* family (Walker et al. 2018). VHSV is divided into four genotypes I-IV and has been described in more than 80 fish species in both fresh and marine waters of the Northern hemisphere (review by Skall et al. 2005a, Elsayed et al. 2006, Lumsden et al. 2007, Dale et al. 2009, Bain et al. 2010, Gadd et al. 2010, 2011, Kim and Faisal 2010, Emmenegger et al. 2013, Ito and Olesen 2013).

VHSV causes heavy losses due to high mortalities in fish farms, especially in rainbow trout farming. The severity of the infection depends on the fish species and virus strain. VHS is a notifiable disease in the European Union. The disease is listed as a non-exotic disease that is prevented and controlled according to *European Council Directive 2006/88/EC on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals* (EC 2006).

Finland joined the European Union in 1995 and started an intensive screening of Finnish fish farms for infectious haematopoietic necrosis IHN, spring viremia of carp SVC, infectious pancreatic necrosis IPN, VHS and bacterial kidney disease BKD, to prove freedom from these diseases. After five years of screening, VHS was diagnosed for the first time in spring 2000 at a fish farm producing rainbow trout for consumption in open net pens in the sea area of the Province of Åland (hereafter called Åland), and almost simultaneously at a similar fish farm on the south coast of continental Finland approximately 330 km away (Raja-Halli et al. 2006). Infection with VHSV genotype Id spread rapidly between fish farms in Åland, despite extensive eradicative measures, and in 2001, a restriction area comprising the whole province was established. Movement of live fish, uncutted farmed fish and fish farming equipment including well boats from the restriction area was forbidden. However, eradicative measures in the second area on the south coast were successful, and VHSV has not been isolated there since 2001. In 2003, VHSV spread from Åland to a third area, to fish farms also producing rainbow trout in the municipalities of Uusikaupunki, Pyhäranta and Rauma, on the west coast of continental Finland (Raja-Halli et al. 2006). VHS was successfully eradicated from this third area in the same year and VHSV was not reported there until 2008, when the virus was isolated again in the same area in a fish farm producing rainbow trout for consumption in Pyhämää. Eradication was repeated and no infection has been recorded since 2008.

The source of the first VHSV infections in Finland in 2000 is still open. There was no known contact between the two fish farms where the first VHS outbreaks were found in 2000. The strongest suspected source of the infection was wild herring. Einer-Jensen et al. (2004) reported in their evolutionary study on isolated VHSV strains that the Finnish isolates from rainbow trout resembled the old Danish isolates. They suggested that the Finnish isolate could have evolved from marine VHSV types similarly to what is believed to have happened in Denmark in the 1930s when the disease first occurred (Einer-Jensen et al. 2004). The diseased fish of the first cases in Finland had not been fed wild herring, but both farms were situated close to harbours for herring trawlers offering close contact with trawled herring.
The fish farming industry in Åland started to improve biosecurity on farms, but keenness to change the infrastructure to a higher biosecurity standard was low, as wild fish were believed to be the source of reinfections. In Åland, new disease outbreaks were often reported, even as soon as 1-2 weeks after fish from a VHS-free area were moved to localities that had been empty of fish for 8–12 months, including the removal and disinfection of all farming equipment (author’s own experience).

Nevertheless, surveillance efforts that screened wild herring, sprat, salmonid brood fish and lampreys (Lampetra fluviatilis) for VHSV during 2004–2006 on the west coast of Finland in the Baltic Sea remained negative for VHSV Id (Gadd et al. 2010, 2011). Furthermore, the subsequent screening of wild fish in the vicinity of the study farms reported on herein also suggested a lack of VHSV in wild fish (Vennerstöm et al. 2018). Recurrent outbreaks of VHS in Åland were difficult to explain and suspicion about the rationale and effectivity of the surveillance programme was raised, including the diagnostic sensitivity of screening for the presence of VHSV infection. It was suspected that the surveillance programme and methods used were only able to find the ‘tip of the iceberg’ and that in order to achieve successful eradication, surveillance activities needed to be improved.

This study tested different surveillance strategies of the farmed fish populations and compared different diagnostic tests for detecting if a population is or had been infected by VHSV. The role of wild fish as reservoir of VHSV was studied. The occurrence of VHSV in the environment like blue mussels, sea water, sediment and wastewater in processing plants handling VHS infected fish were also studied.
2 Review of the literature

2.1 Fish farming and monitoring of fish diseases in Finland

Farming of fish has been practised in Finland since the mid-19th century. In the beginning, eggs were collected from wild brood stock along the main salmon rivers and hatched to restock the natural fish resources. Commercial fish farming started in the late 1950s when salmonids were hatched and farmed in ponds. Fish farming became more intensive in the 1960s when rainbow trout (Oncorhynchus mykiss) production grew. Rainbow trout, a fish species originating from the Pacific coast of North America, was imported to Europe in the late 19th century, and shortly after this even to Finland (EC 2012, Finnish Fish Farmers' Association 2019).

The number of fish farms increased, reaching its peak in the early 1990s when the environmental authorities started restricting farming activities by making licensing stricter. Fish farming is a highly restricted and regulated activity in Finland, ensuring the well-being of the environment and fish. Today any fish farming activities that on a yearly basis produces more than 2000 kg fish or uses more than 2000 kg fish feed needs to be licensed according to the Environmental Protection Act (Ministry of the Environment 2014). Additional licensing is needed for building water sources according to the Water Act (Ministry of the Environment 2011) and all fish processing activities need to be licensed. Health authorisation is also acquired for the sale or transfer of fish for food, on-growing or restocking from farms or from ponds with natural nutrition (EC 2014).

Fish are farmed for two main reasons in Finland: producing food fish and restocking the sea, rivers and lakes. Fry for food fish production are mainly received from broodfish farmed in inland farms using natural water from lakes and rivers or well water. The hatcheries consist of a hatchery section and a grow-out section for the newly hatched fry. Fry are usually moved to out-door ponds for further growing before they are sold to food fish producers, which are mainly situated in the sea area where food fish are farmed in net pens that are anchored to the bottom or to the nearby shore.

Companies that farm food fish in the sea area have separate localities for farming during summer and winter. The coast of Finland has quite shallow waters and the conditions for farming salmonids are not optimal regarding water temperature and oxygen levels during summertime. Winter localities are often located in shallow bays or by surrounding islands close to the mainland where they are protected from harsh weather conditions in autumn, and especially in winter when the sea may be covered by ice. During summer, water temperatures often rise over 20 °C in the winter localities and fish are moved to the summer localities situated in cooler and deeper waters more optimal for farming rainbow trout. The farming licences also restrict the amount of fish that may be placed in winter localities and order that fish must be moved to summer localities before a certain date. Fish that have reached slaughter size in autumn are often moved as close to the processing plants as possible or transported by well boats directly from the summer localities for processing. Fish that will not attain slaughter size until the next year are often kept in the winter farming localities for the first year.
Processing plants are usually the place where the fish food and farming equipment are stored, including boats for the daily servicing of the farming localities. One food fish producing company in the sea area usually has several farming localities that are scattered around the many islands of the archipelago (see map in figure 1, Vennerström et al. 2017 (I)). There are often daily contacts between most of the farming localities and the processing plant. The winter farming localities are also often situated next to the processing plant.

Rainbow trout has been the main farmed species for food production, but in the 2000s the farming of whitefish (*Coregonus lavaretus*), a native fish species, began in the sea and inland waters. In addition, other native fish species are farmed for food mainly in the inland area, like arctic char (*Salvelinus alpinus*), pike perch (*Sander lucioperca*) and sea trout (*Salmo trutta trutta*) at sea. Sturgeon (*Acipenser sturio*) that is originally imported to Finland is also farmed in a few farms in the inland area (Finnish Fish farmers' Association 2014, Finnish Fish Farmers' Association 2017).

In 2018, the production volume of domestic food fish was 14.3 million kg, about 73% of the peak production year of 1991 (19.6 million kg) (Figure 1). About 83% (11.9 million kg) of the farmed food fish are produced in the sea area, of which 60% (approx. 7.1 million kg) are produced in the Åland Islands, a province on the south-west coast of Finland. In 2018, altogether 13.2 million kg of rainbow trout was produced, which is over 90% of the whole of the food fish production in Finland. In addition, 0.8 million kg whitefish was produced (Natural Resources Institute 2019).

![Food fish production (ungutted fish) and its value (deflation: cost-of-living index)](image)

*Source: OSF: Natural Resources Institute Finland, Aquaculture.*

**Figure 1.** Annual amount of produced food fish in Finland, 1980-2018 and its value. Figure obtained from Aquaculture Statistics of the Natural Resource Institute Finland (National Resources Institute 2019).
Restocking of fish has been an important way of ensuring natural resources, especially in waterways where natural spawning migration from the sea is blocked by hydro-power plants. Progeny are collected either from broodfish kept in inland fish farms or from wild-caught broodfish living in either inland water systems or from the sea area. Fertilised eggs are hatched and the fry either moved for further growing in artificial ponds or to a pond with only natural nutrition. Due to the prevention of certain fish diseases spreading from the sea area to inland waters, live fish material (mainly eggs) from the sea may only be brought to the inland area via quarantine. The broodfish, from which the eggs originated, are monitored for certain fish diseases and the eggs are transported into inland waters only if the test results are negative. When fry have gained the desirable size, they are released into the water system to improve the natural resources. Greatest numbers of restocked fingerlings are those of whitefish (Coregonus lavaretus) and pike perch (Sander lucioperca). The value of the stocked smolts of Atlantic salmon (Salmo salar), sea trout (Salmo trutta trutta) and brown trout (Salmo trutta lacustris) is, however, much higher. Some other species like arctic char (Salvelinus alpinus) are farmed for this purpose, too.

The prevention of fish diseases in cultivated fish has quite a long history in Finland. A voluntary fish health surveillance programme was established 50 years ago in 1969. This programme was run by the former national veterinary institute (today Finnish Food Authority), has evolved over the years to fit its purpose and is still available for all fish farmers in Finland (Finnish Food Authority 2019). The main aim of the programme has been to ensure that the top of the production pyramid (broodfish and hatcheries) stays as free from fish diseases as possible. This has been well-achieved by categorising fish farms depending on where in the water system they are situated and what kind of production they have. Movement of fish is strictly directed from the top of the pyramid downwards. The laboratory (today the Finnish Food Authority) and the farmer make an agreement about the programme, in which the laboratory agrees to offer diagnostics for fish diseases and give advice to the farmer and local veterinarians regarding health issues on the farm. The farmer agrees to pay an annual fee and inform the laboratory about noticed or suspected fish diseases on the farm by sending samples for diagnostics. The annual fee includes testing of samples in connection to disease issues. The programme has always been voluntary and the coverage of the farms participating in the programme differs depending on type of production. Almost all farms producing progeny (broodfish farms, hatcheries and producers of fry) participate. Among the food fish farmers, the participation has always been quite poor. Screening of notifiable fish diseases was included in the health surveillance programme until Finland joined the European Union (EU) in 1995. After this, screening of notifiable fish diseases was included in the compulsory surveillance programmes issued by the EU. There are no fish health veterinarians working in the field in Finland and the official sampling for screening of notifiable fish diseases is performed by local municipal veterinarians. Although these veterinarians often have minor skills in fish diseases, some gain knowledge in the field during several years of experiences. Three universities perform research on fish health issues, mainly parasitology and bacteriology, and some offer some diagnostic services for fish farms. Fish virus diagnostics has been performed in the Finnish Food Authority national reference laboratory since 1969.
The disease situation in Finnish fish farms has been quite good. In the beginning, bacterial infections like *Vibrio anguillarum* and *Aeromonas salmonicida sp. salmonicida* caused heavy losses and the use of antibiotics was very high (Figure 2). In the mid-1990s, effective vaccines against these two bacterial infections became available and the use of antibiotics in food fish farming decreased dramatically. Today these two bacterial diseases are rare in vaccinated fish. A slight increase in the use of antibiotics was noticed again in 2010-2011 when biotype 2 *Yersinia ruckeri* appeared in food fish farms in the sea area but decreased when vaccination against yersiniosis was started (Figure 2).

Infections with flavobacteria are the main disease problem today and cause high losses in the production of fry, also affecting fish health in the sea area. Commercial vaccines against *Flavobacterium psychrophilum* were available for a few years but were withdrawn from the market as farmers did not use them (author’s own experience). The stumbling block for the development of useful vaccines against flavobacterial infections is that the infections are severe and start from newly hatched fry that cannot be vaccinated.

The food fish farming industry faced one of their biggest challenges in 2000 when viral haemorrhagic septicaemia (VHS) was for the first time found in two separate food fish farming areas on the south-west and south coasts of Finland (see section 2.4.2). Another drawback in the disease situation was when IPN was found in 2012 in the inland area that had been IPN-free (Eriksson-Kallio et al. 2016). Eradication of IPN-positive farms was considered impossible and because the IPN isolates were of the less pathogenic genogroup 2, eradication procedures were withdrawn. Inland area is still free from IPN of genogroup 5. A third alerting disease event came in 2017 when infectious haematopoietic necrosis virus (IHNV) was for the first time isolated in sea-reared rainbow trout in two food fish farms on the north-west coast of Finland. In the epidemiological survey of the contact farms, a total of six farming localities were IHNV-positive, two of which were considered possible sources for the other infections. The infected farms have been eradicated and today the infected areas are under surveillance before they can be declared IHN-free compartments again (Finnish food safety authority 2018, Finnish Food Authority 2019).
2.2 Viral haemorrhagic septicaemia

2.2.1 The disease

In 1938 Schäperclaus described a new serious disease of rainbow trout in Germany that he later termed *kidney swelling and liver degeneration* (Jensen 1965). He described a syndrome with symptoms of acute septicaemia of unknown aetiology that spread among rainbow trout farms and became a serious disease for the trout industry in continental Europe (Schäperclaus, 1954). During winter 1949-1950, a similar syndrome was detected in farms producing rainbow trout in Denmark in a small village called Egtved. Jensen from the state serum laboratory managed to isolate the causative agent for the first time in 1963, a virus that was later confirmed as a rhabdovirus (Jensen 1963, Zwil敏捷berg 1965). The virus was named Egtved virus after the village of the first isolation. Several names had been used for the syndrome among farmers and scientists and Egtved disease became a common name for the disease, although the disease was later named Viral haemorrhagic septicaemia (VHS) by an international agreement in 1963 (Wolf 1988).

The onset of an outbreak of VHS is characterised by nonspecific clinical signs that are followed by rapidly growing mortality. In rainbow trout three different steps of VHS can be seen; acute, chronic and nervous form (Smail 1999). The first disease signs in a VHSV-infected rainbow trout population is that they stop feeding and become lethargic (Wolf 1988). The diseased fish are found close to the edge of the pond or cages where the water is still, and some fish drift against the sieve of the outlet drain as they cannot fight the water current (Wolf 1988). The diseased fish population may get easily scared by movement
above the water surface, making them rush to the edges of the ponds or net pens (Wolf 1988). Diseased fish have a darker pigmentation of the skin, swim erratically and have difficulty in orientation. Bulged eye (exophthalmia) (Figure 3), with haemorrhage around the eye orbit, can be seen in one or both eyes (Wolf 1988). Gills are pale due to severe anaemia (Figure 3). Internally the most pronounced changes can be seen in the kidney and liver (Smail 1999). The kidney is swollen and dark red in the early stages, but later the front and midsection are pale due to necrosis of haematopoietic tissue. The liver is pale and yellowish with areas of mottled haemorrhage and focal areas of necrotic hepatocytes. The most pronounced visible signs in the acute stage are widespread petechial haemorrhages that form as the virus multiplies in the endothelial cells of the capillaries and causes haemorrhages in several location of the body, i.e. the eyes, visceral fat, peritoneum, swim bladder, kidney, liver, skeletal musculature (Figure 3) and heart muscle (Smail 1999). In survivors of the acute stage, haemorrhaging is reduced, but anaemia is severe. Nervous signs are common in the chronic stage: corkscrew-like swimming and some fish may hang in a candle-like position with the head towards the surface and the tail down. The disease signs are not pathognomonic for VHS and may be seen in several other severe septic infections of both viral and bacterial origin. Surviving fish or wild fish that are not sensitive to VHSV are suspected to become carriers of the virus (Schönherz et al. 2013, OIE 2019).

Mortality in VHS-infected rainbow trout varies from mild to severe depending on the virus type, age, stress and water temperature (Smail 1999). VHSV isolates that origin from European freshwater-reared rainbow trout are highly pathogenic to rainbow trout but not to marine fish species (Skall et al. 2004a). Small rainbow trout fry (0.3-3g) have been most susceptible to VHS, as they possess proportionally more of the target organ tissues for the virus than older fish and can reach mortalities up to 80-100% (Smail 1999). Fingerlings and growers are also susceptible, but mortalities are often lower, at 10-50% (Smail 1999). The optimum temperature for virus replication has been estimated from infection trials to be 9-12 °C (Smail 1999, Goodwin and Merry 2011). The highest mortalities are seen in spring when the water temperature is fluctuating. Virus replication is decreased in temperatures over 15 °C (OIE 2019, Avunje et al. 2012). Stress such as handling can reactivate the VHS infection of a population in a carrier state (Hoerylyck et al. 1984, Olesen 1998).

In other farmed fish species like Japanese flounder (Paralichthys olivaceus), turbot (Scophthalmus maximus) and sea bass (Dicentrarchus labrax), symptoms like those in rainbow trout have been described in natural infections of VHSV (Takano 2000, Ishiki 2001). A common characteristic is widespread petechial bleeding in the external and internal organs even though the severity of the bleeding varies in different fish species. VHSV has caused mass mortalities with typical signs of VHS in several wild fish species in North America such as Pacific herring (Clupea pallasi) and muskellunge (Esox masquinongy). In some species, like cod (Gadus morhua), rockling (Rhinonemus cimbrius) and haddock (Melanogrammus aeglefinus), VHSV has been isolated from skin ulcers (Jensen 1979, Mortensen et al. 1999, Snow 2000, Smail 2000).
2.2.2 VHS virus

The causative agent of viral haemorrhagic septicaemia is viral haemorrhagic septicaemia virus (VHSV), which is a Piscine novirhabdovirus classified into the genus of Novirhabdovirus belonging to the family Rhabdoviridae and Mononegavirales order (Walker et al. 2018). Rhabdoviridae include 18 genera of which 12 infect animals including mammals, birds, reptiles and fish; the remaining 6 genera are arthropod and plant viruses (Walker et al. 2018). One of the most well-known is Lyssavirus, which infects several mammals including humans in which they cause fatal encephalitis (rabies) (Walker et al. 2018). Other genera of the Rhabdoviridae family that infect fish are Perhabdovirus and Sprivivirus (Walker et al. 2018). The Novirhabdovirus genus include another three species Hirame novirhabdovirus, Salmon novirhabdovirus and Snakehead novirhabdovirus (Walker et al. 2018). Rhabdoviruses are RNA viruses with bullet-shaped virions (Figure 4) containing a negative-sense, single-stranded RNA (ssRNA) genome (Walker et al. 2018). The virion of VHSV is approximately 70 nm in diameter and 180 nm in length (Kimura et al. 1986, Wolf 1988, Kasornchandra et al. 1992). Like other rhabdoviruses, VHSV has a lipid envelope with glycoprotein (G) anchored to the membrane by a N-terminal hydrophobic transmembrane region. The glycoproteins at the surface of the virion act as the receptor-binding ligand and target of neutralizing antibodies. VHSV isolates can all be identified by the monoclonal antibody (MAb) IP5B11, making the virus serologically homogenous in this respect (Lorenzen et al. 1988). The genomic RNA sequence of VHSV comprises 11,158 bases and contains six genes in the order 3’-N-P-M-G-NV-L-5’ (Figure 5). The genome begins with a non-coding 3’ leader sequence and terminates with a non-coding 5’ trailer sequence. One gene encodes a non-virion protein (NV) (12-14 kDa) (Kurath and Leong 1985, Schutze et al. 1999) and five genes encode structural proteins: the nucleocapsid- (N) (size: 38–47 kDa), phospho- (P) (22–26 kDa, formerly designated M1), matrix- (M) (17–22 kDa, formerly designated M2), glyco- (G) (63–80 kDa) and RNA polymerase protein (L) (150–225 kDa) (Lenoir and de Kinkelin 1975, McAlister and Wagner 1975, Leong and Kurath 2012). The presence of the non-virion gene (NV), located between the G and L genes in the genome, distinguishes members of the genus novirhabdovirus from other rhabdoviruses. NV protein is detected in
the nucleus of infected cells but not in virions (Schutze et al. 1999, Choi et al. 2011) The expression of the gene is polycistronic, i.e. each gene is translated from a separate mRNA which are transcribed in a gradient fashion, e.g. more abundant from “left to right” (more mRNA and thus more protein N and least of L) (Maclachlan et al. 2011).

Figure 4. Electron micrograph showing budding of the fish novirhabdovirus infectious haematopoietic necrosis virus at the plasma membrane and characteristic bullet-shaped rhabdovirus virion structure. Reprinted from Granzow et al. (1997) (Figure 2F) with permission from John Wiley and Sons, Publisher.

![Figure 5](image)

**Figure 5.** Schematic representation of novirhabdovirus genome organisations. N, P, M, G and L represent ORFs encoding the structural proteins. ORF NV (U1), encoding a protein involved in pathogenicity and blocking host innate immunity, is highlighted (red). (Reprinted from ICTV 10th report)
2.2.3 Pathogenesis

To be able to understand the epidemics of VHS, it is important to know how the causative agent manages to develop the disease in its host: (1) survival of the agent in the environment, (2) port of entry of the virus to the fish, (3) replication of the virus in the host cell, (4) transmission of the virus, (5) encountering the host’s immune system and (6) escaping the immune system of the fish.

Virus survival outside the host depends on physico-chemical conditions in the environment (Bovo et al. 2005a, 2005b). VHSV is more stable in cold water temperatures (4°C) than in warm (20°C). In cold water, VHSV can survive from a few days in natural fresh or sea water and up to a year in filtered freshwater (Parry and Dixon 1997, Hawley and Garver 2008). Freshwater seems to be more favourable for virus survival than sea water according to Hawley and Garver (2008). Rhabdoviruses like VHSV and IHNV are sensitive to UV irradiation (Øye and Rimstad 2001, Yoshimizu et al. 1986).

There are several sequential studies on the pathogenesis of VHS in different fish species experimentally infected by VHSV. These studies indicate that horizontal transmission of virus particles through water is the main transmission route (Stone et al. 1997, Snow et al. 2005, Kurath and Winton 2011). The main entry route into fish challenged by bath is via the gill epithelium and the virus is further transported via the blood to the main internal organs (Smial 1999, Brudeseth et al. 2002a, 2005, Matras et al. 2006). Evensen et al. 1994 found VHSV antigen in rainbow trout endothelial cells of the anterior kidney as early as 2-4 days post-infection. Hepatocytes and exocrine pancreatic cells were also infected less than a week post-infection. The virus starts replicating and causes damage to the endothelium in the circulatory system, as damage in all these organs was noticed to start from day 4. Skin has also been reported as a likely route for virus entry and a site for early replication (Yamamoto et al. 1992). Moreover, an oral infection route has also been reported by feeding infected fish or feed to susceptible hosts such as pike and rainbow trout (Ahne 1980, Meyers 1995, Schönherz et al. 2012).

**VHSV replication and transmission**

The replication of rhabdoviruses is presented in Figure 6. VHSV enters the host cell by endocytosis that is receptor mediated. The endocytosis is triggered by the viral G protein that is located at the envelope of the virus and binds it to a fibronectin complex (Bearzotti et al. 1995, Assenberg et al. 2010, Purcell et al. 2012). The cell membrane and virus membrane fuse and the viral nucleocapside is released into the cytoplasm of the host cell (Assenberg et al. 2010, Purcell et al. 2012). The infective virion contains an RNA-directed RNA polymerase that transcribes viral genes and uses the host cell to synthesise viral proteins. First a full length single-stranded positive-sense RNA (complementary to the genomic RNA) is produced to generate copies of negative-sense genome RNA for the new virions. The N, L and P proteins are synthesised by free ribosomes and bind to the newly formed viral RNA genome, building up the ribonucleoprotein (RNP) core. The RNP further forms a complex with the M protein. The G protein is translated by ribosomes at the
endoplasmatic reticulum with subsequent posttranslational modifications occurring at endoplasmatic reticulum and Golgi apparatus during the vesicular transport to the plasma membrane of the host cell. The RNP-M complex migrates beneath the plasma membrane enriched with G proteins and promotes budding of enveloped infective virions (Purcell et al. 2012). The virus is spread by urine from diseased fish (Wolff 1988). Vertical spread of VHS virus is not reported and disinfection of eggs with iodine has prevented infection (Olesen and Skall 2013). Significant amount of VHSV may be present in the tissues of processed rainbow trout originating from an infected population and could thereby be transferred to VHS-free areas (Oidtmann et al. 2011a).

**Figure 6.** Virus replication of rhabdoviruses is divided into four main parts. 1 Phagocytosis, virus binds to the cell and enters. 2 Uncoating, viral genome and polymerase is released by fusion of the glycoprotein and matrix membrane. 3 Transcription of a positive-sense RNA that is translated into viral proteins by the endoplasmic reticulum (ER) and Golgi apparatus. 4 The positive-sense RNA is replicated back to a negative-sense RNA and viral components assembled for release of new virions through budding (Picture: reprinted from Schnell et al. 2010) (with permission from Springer Nature)

**Immunity in fish**

Immunity is usually divided into two different strategies in fish, as in other vertebrates: innate immunity that is the unspecific ‘first-line defence’ in infections and specific humoral immunity that builds up a long-term adaptive immunological memory. Innate immunity consists of the physical barrier and cellular response. The cellular responses in innate immunity are well-described in mammals in which pathogenic molecules are recognised by pattern recognition receptors (PRRs) that lead to the activation of immune effector
molecules like the interferon (IFN) system (Kawai et al. 2008). Immunity against viral nucleic acids, including those of rhabdoviruses, is induced by Toll-like receptors (TLR) 3, 7 and 8 or the retinoic acid-inducible gene 1 (RIG-1) and melanoma differentiation-associated gene 5 (MDA5) (Mogensen and Paludan 2005). Teleost fish recognise viral nucleic acids of rhabdoviruses in almost the same pattern as mammals with some exceptions (Zou et al. 2010). They have in addition a set of non-mammalian TLR genes (Rebl et al. 2010, Palti, 2011). Fish also possess genes encoding cytokines, chemokines and other innate effectors for producing type 1 interferons (IFN) that are expressed in several different tissue types and type II IFN that is mainly expressed in haematopoietic tissues and cells (Altman et al. 2003, Long et al. 2004, Long et al. 2006, Robertsen et al. 2003, Sun et al. 2009, Zou et al. 2007, Milev-Milovanovic et al. 2006, Zou et al. 2005, Stein et al. 2007).

The humoral immune response produces neutralising antibodies that are crucial components in immunity against fish rhabdoviruses and the antibody response in teleost fish (reviewed by Lorenzen and LaPatra 1999). Fish possess several immunoglobulin types IgM, IgD, IgZ IgT and a polymeric Ig receptor pLgR that function as an immunoglobulin transporter to mucosal surfaces (Hansen et al. 2005, Danilova et al. 2005, Hordvik et al. 2002, Zhang et al. 2010, Rombout et al. 2014, Bengten et al. 2015,). There are indications that B-cells expressing IgM respond to antigenic stimuli in systemic fish tissues whereas B-cells with IgT function on the mucous membranes (Zhang et al. 2010, Yu et al. 2019). The role of IgT in fish rhabdoviral infections is not known.

It has recently been demonstrated that many of the cells and molecules that have been considered unique to either the innate or adapted immune system actually function in both, making the communication between the systems more complex than earlier believed (Abós et al. 2015, Secombes, 2016).

Cells involved in the immunity of fish are T and B lymphocytes, natural killer cells, monocytes, macrophages, neutrophils, eosinophils, mast cells and thrombocytes (Castro and Tafalla 2015). In addition, dendritic cells have been described in Atlantic salmon (Salmo salar) and rainbow trout (Fuglem et al. 2010, Haugland et al. 2012, Johansson et al. 2012). Fish probably also possess specific cellular immunity, as high levels of specific protection have been recorded in DNA vaccination trials using G protein of novirhabdovirus, although no neutralising antibodies were detected (Lorenzen et al. 1998, LaPatra et al. 2000). Little is known about the role of fish T-cells in infections with fish rhabdoviruses, but some evidence of cell-mediated cytotoxicity (CMC) has been described in host responses to this group of fish viruses (Somamoto et al. 2002, Utke et al. 2008).

Fish are poikilotherm organisms whose physiological functions are slower in water temperatures that are suboptimal for certain fish species. Temperature affects the function of the immune response and virus replication in fish. Immunological suboptimal temperatures lead to an insufficient immunological response due to the slower immune response (Le Morvan et al. 1998). VHSV infections are mostly detected in temperatures below 15 °C, and mortality in low temperatures is usually higher (Wolf 1988). Fish that have survived a VHSV infection clear the virus below the detection level at optimal temperatures and develop good protective immunity. The development of specific immunity is delayed in fish that are kept in low temperatures and the role of innate immunity becomes crucial (Le Morvan et al. 1998, Alcorn et al. 2002, Lorenzen et al. 2009). Low water
temperature is also suggested to be a factor for persistent rhabdoviral infections, probably due to suppression of the humoral adaptive immune response. Neukirch (1986) reported that VHSV was detectable in the brain for 400 days post-infection in rainbow trout held at 4 °C without any clinical signs of infection or detectable antibodies in serum. Another study on VHSV infections in Pacific herring suggested that it is not clear if individuals in a population become carriers of the virus or if the virus persists in the population by jumping between naïve or convalescent hosts (Hershberger 2010, Purcell 2012). Other factors that have been indicative of affecting the immune response are diet, age, seasonality and reproductive status, not to forget stress (Sealey et al 2007, Utke et al. 2008, Beaulaurier et al. 2012, Martin and Król 2017).

**Immune evasion of rhabdoviruses**

The way in which viruses ensure survival and transmission can be divided into two main strategies: ‘hit and run’ or ‘hit and stay’ (Hilleman 2004). Cytolytic viruses like VHSV are mainly considered ‘hit and run’ viruses as they destroy the infected cell and are highly infective and ready to transmit to new hosts before the host’s cell-mediated immunity stops them or the host dies. ‘Hit and stay’ viruses ensure they can stay in the host by escaping the host’s immune system in a way that ensures virus survival in the host for a longer period, even for good. VHSV may use both strategies depending on the immune status of the host, as varied forms (acute, chronic and nervous) of the disease are well-described. In addition, persistent infection with an asymptomatic carrier state has been described (Vestergård Jørgensen 1982, Neukirch 1986). VHSV is sensitive to the effects of IFN, but virulent rhabdovirus strains can continue replicating in the host despite IFN, as they possess several different mechanisms to evade the host’s immune system (Ahmed et al. 1998). They may directly interfere with the interferon system, e.g. the M protein is able to mediate cell shut-off in infectious haematopoietic virus (IHNV) infections (Chiou et al. 2000). Cell shut-off is a cascade of reactions interfering with the programmed cell death process (apoptosis) that host cells use to get rid of virus-infected cells before the pathogen can multiply in the cell (Ahmed et al. 1998, Lyles 2000). Cell shut-off has so far not been described for other fish rhabdoviruses than IHNV (Purcell 2012). The NV protein of Novirhabdoviruses also participates in the immune evasion of the virus by preventing apoptosis and interfering with the unspecific innate immune response of the host (Ammayappan et al. 2011, Kim and Kim 2013, Biacchesi et al. 2017). NV can also depress the IFN response in fish, but indication of this has only been shown for IHNV (Choi et al. 2011). The NV protein is required for effective virus replication and is suspected to be an essential contributor of the pathogenicity of VHSV and IHN (Biacchesi et al. 2017, Johnson et al. 2000, Thoulouze et al. 2004, Ammayappan et al. 2011). Antigenic escape by antigenic drift in the G gene (virus evolution) induced by immune selection is not clearly demonstrated, although there are results indicating that it may happen (Huang et al. 1996, Troyer et al. 2000, Kurath et al. 2003, Purcell 2012).
2.3 VHSV strains, hosts and geographical distribution


VHSV group I includes five sublineages (a, b, c, d, e) of which ‘a’ represents European freshwater isolates and isolates from sea-reared rainbow trout and turbot (Schlotfeldt et al. 1991, Snow et al. 2004, Einer-Jensen et al. 2004, Toplak et al. 2010). VHSV sublineage ‘a’ can further be divided into two major subpopulations, Ia-1 and Ia-2 (Kahns et al. 2012). VHSV sublineage ‘b’ strains originate from the Baltic Sea, Skagerrak, Kattegat, the North Sea, and the English Channel and there has been one case in Japan (Snow et al. 2004, Einer-Jensen et al. 2004, Skall et al. 2005b, Nishizava et al. 2002, Nordblom and Norell 2000). Sublineage ‘c’ includes older Danish freshwater isolates from rainbow trout and has also been reported in Germany and Austria (Jonstrup et al. 2009). Sublineage ‘d’ includes Scandinavian isolates from the 1960s (Olesen and Skall 2013) and isolates from rainbow trout farms in brackish water in Finland (Raja-Halli et al. 2006). Sublineage ‘e’ is a single isolate from Georgia (Kalayci et al. 2006, Nishizava et al. 2006, Jonstrup et al. 2009).

Group II includes strains isolated from wild fish in a small region close to Gotland and from the west coast of Finland, mainly herring (Clupea harengus) and some isolates from lamprey (Lampetra fluviatilis) (Einer-Jensen et al. 2004, Gadd et al. 2010, 2011).

Group III includes isolates from both wild and farmed fish in the North Sea close to the UK and Ireland as well as Norway (Snow et al. 1999, King et al. 2001, Skall et al. 2004b, Dale et al. 2009). The group includes several distinct sublineages that have not been named separately, apart from the Norwegian isolates from 2007 that have been suggested to be named IIIb since they differ genetically from earlier isolated strains (Dale et al. 2009).

Group IV includes three sublineages (a, b, c), of which ‘a’ consists of North American isolates from wild marine and anadromous fish along the Pacific coast (Meyers and Winton 1995, Marty et al. 1998, Hedrick et al. 2003). Sublineage ‘b’ is isolates from the Great Lakes watershed of the United States and Canada (Elsayed et al. 2006, Groocock et al. 2007, Lumsden et al. 2007, Ammayappan and Vakharia 2009). Strains of sublineage ‘c’ have been found on the east coast of North America (Pierce and Stepien 2012a, 2012b) Genotype IV isolates have also been found in Japan and Korea (Nishizawa et al. 2002, 2006, Kim et al. 2003, Ito and Olesen 2013). In 2015 VHSV was isolated from wild lumpfish (Cyclopterus
lumpus) that was caught to be use as broodfish in Iceland. This isolate differs from earlier reported genotype IV strains and has been suggested to be a novel subgroup for the genotype IV (Guðmundsdóttir et al. 2019).

Rainbow trout (Oncorhynchus mykiss) is one of the most sensitive farmed fish species to VHSV genotype I, but turbot is also reported to be sensitive to Ie, III and IVa (Skall et al. 2005b). VHS has also caused severe disease in farmed Japanese flounder (Paralichthys flesus) (Isshiki et al. 2001). Extensive mortalities in wild fish due to VHSV genotype IV have been reported in North America in Pacific herring (Clupea pallasiid) on the Pacific coast and in up to 28 different wild fish species in the Great Lakes watershed and on the east coast (Marty et al. 1998, Meyers et al. 1999, Gagné et al. 2007, Elsayed et al. 2006, Lumsden et al. 2007, Groocock et al. 2007, Ammayappan and Vakharia 2009).

According to phylogenetic studies, VHSV has its ancestors in the marine environment, from where it has adapted to be a serious disease agent for farmed rainbow trout (Oncorhynchus mykiss) (Einer-Jensen et al. 2004). The ‘change in host range’ has probably happened several times since the first reports of clinical outbreaks of viral haemorrhagic septicaemia (VHS) originating from the 1950s (Einer-Jensen et al. 2004). These leaps over species barriers are believed to be a result of human activities in connection to fish farming procedures. Wild marine fish, mainly herring (Clupea harengus), were intensively used as minced fresh feed for freshwater farmed rainbow trout in Europe in the 1950s (Meyers and Winton 1995, Dixon 1999, Einer-Jensen et al. 2004). The use of fresh marine fish ceased throughout Europe when several wild marine fish species, including herring, were found to be carriers of VHSV and it was found that it could be spread by the oral route (Stone et al. 1997, Snow et al. 1999, Mortensen et al. 1999). VHSV types pathogenic to rainbow trout have been isolated from wild fish in Europe on several occasions, but mostly no mortality or clinical signs of VHS have been reported in these wild fish species (Skall et al. 2005b).

After VHS was found in marine fish and severe outbreaks in sea-reared turbot were reported from Germany, Scotland and Ireland, wide research programmes to screen wild fish for VHSV were conducted. Screening was performed by Danish, Norwegian and Scottish institutes covering the coastal waters of Scotland, the North Sea, and the coastal waters of the west and south of Norway, Skagerrak, Kattegat and the Baltic Sea (Skall 2005b). They examined a total of 54 137 fish representing 63 different fish species. VHS virus was found in 193 samples from 15 fish species (Mortensen et al. 1999, Smail 2000, King et al. 2001, Brudeseth and Evensen 2002b, Dixon et al. 2003, Skall et al. 2005a). VHSV was found to be endemic in the Baltic Sea, Kattegat, Skagerrak, the North Sea and around the British Isles (Skall 2005a). The prevalence of VHS virus was highest in waters around Bornholm, with prevalence ranging from 0-16.7% for herring and 5.6-7.8% for sprat (Sprattus sprattus) (Skall 2005a). Similar prevalence was found in the waters close to California and Oregon in clinically healthy sardine (Sardinops sagax) and mackerel (Scomber japonicus) (Hedrick et al. 2003).

In Finland, wild salmonid brood fish, Baltic herring (Clupea harengus membras L.) and lamprey (Lampetra fluviatilis) were screened for VHSV in the coastal waters of the south-west coast of Finland around the area in the Baltic Sea where VHSV-positive (genotype Id) rainbow trout farms are located. VHSV Id was not isolated but Baltic herring and lamprey were found to be carriers of VHSV genotype II (Gadd et al. 2010, 2011). The highest
prevalence of VHS II, 50/479 pools (10.4%), was recorded in herring originating from the Archipelago Sea south-west of Finland (Gadd et al. 2011). However, VHS has never been recorded in farmed fish in this high-prevalence area, even though intensive fish farming has been performed for decades and minced wild herring was earlier used as fresh feed. According to the infection trials, the pathogenicity of the isolated VHSV genotype II strains to rainbow trout was negligible (Gadd et al. 2011).

2.4 Prevention and control of VHSV

VHS is a notifiable disease in the European Union (EU) and one of the diseases listed by the World Organisation for Animal Health (OIE) (OIE 2019). In the EU, VHS is classified as a non-exotic but serious fish disease the spread of which must be prevented according to European Directive 2006/88/EC on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals (EC 2014). In the EU, VHS-free areas have been established by monitoring programmes. Aquaculture areas are classified according to their disease status, and movement of live fish is restricted depending on this status.

Finland received VHS-free status (EC 2005) for the continental areas i.e. freshwater systems, in 2005 after a 10-year period of an EU-approved disease monitoring programme. During this programme, all fish farms were inspected once or twice a year by the competent authority and tested for VHSV every year or every second year depending on the production system. Hundreds of inspection visits and a total of 150,000 samples of farmed and wild fish (mainly salmonids) were screened for VHS during these years (EC 2005).
2.4.1 Diagnosing VHS

Typical clinical signs for VHS are an elevated mortality with severe signs of a septicaemic infection (see section 2.2.1). The visible pathological signs are not pathognomonic for VHS, but a rise in mortality in connection to a water temperature below 15°C should always raise a suspicion of a serious virus infection and lead to sampling of diseased fish. EU and OIE have given detailed instructions concerning the surveillance and diagnostics of certain diseases of aquatic organisms including VHS (EC 2015, OIE 2019). Briefly, if a suspicion of VHS is raised, the farmer is obliged to inform the authority who inspects the farm and takes a minimum of 10 diseased fish for virological diagnostics. The fish should be packed in a thermo box with coolers, as the fish must be kept cool during transport. Fish should be in the lab for further processing not later than 48 h post-euthanasia. Sampling is then performed depending on the size of the fish. If the fish length is less than 4 cm, the whole fish is minced after the removal of the body posterior to the gut opening. From fish sized 4-6 cm, the viscera including kidneys should be collected. Larger-sized fish should be sampled from the spleen, head kidney, heart and brain. Samples from no more than 5 fish should be pooled in case of clinical suspicion. Samples are homogenised in Eagles minimum essential media (MEM) and kept on ice during the process. The homogenate is centrifuged, and supernatant is tested with real-time RT-PCR for VHSV RNA (Chico et al. 2006, Matejusova et al. 2008, Jonstrup et al. 2013, Hoferer et al. 2019). In addition, a parallel sample is inoculated into BF-2 and EPC cell cultures to gain the virus strain that can be further sequenced (genotyped) if necessary or just confirm the presence of VHSV by a molecular or antibody-based antigen detection method, e.g. ELISA or IFAT. The sending of whole fish samples is justified as one may also rule out other diseases such as bacterial septicaemias, like infections by Yersinia ruckeri or Flavobacterium psychrophilum that cause symptoms that are difficult to differentiate from VHS and are common in water temperatures below 15 °C. It is very important to be able to either confirm or rule out the presence of notifiable diseases as quickly as possible, as the farm is probably under restriction and waiting for results may cause heavy economic losses and mental stress to the farmer during the testing.

Other diagnostic alternatives are also available that could be used when agent detection is not possible. Serological tests may be useful when the water temperature is too high for virus detection. Antibodies against VHSV take 3-4 weeks to appear in the serum after the infection, but high antibody levels may persist for more than 6 moths (Fregrenda-Grandes and Olesen, 2007, Lorenzen and LaPatra 1999). There is no commercial serologic VHSV antibody test available and an evaluation of tests’ diagnostic sensitivity, specificity and reproducibility is still needed. Therefore, serology alone is still not recommended to be used in VHSV diagnostics (OIE 2019). Another method to show the presence of VHSV is immunohistochemistry, which could be used to raise a suspicion of VHSV infection but needs confirmation by other methods (Evensen et al.1994).
2.4.2 Epidemics and disease management

VHS has caused severe problems in rainbow trout farming in several countries in Europe due to economic losses and trade restrictions. In Denmark, VHSV hampered the trout industry starting from the early 1960s when approximately half of its 800 farms were considered infected by VHSV (Olesen et al. 2013). The losses due to the disease were serious, as the industry could not in the long run deal with the situation. An eradication programme including stamping-out of positive populations, strict biosecurity, regular clinical inspections with sampling of fish populations on farms and trade regulations between different areas with different disease status were established. The programme was based on voluntary participation and the costs of the programme were paid by the industry. The measures proved to be effective and the number of VHSV-infected farms decreased from 400 to approximately 100 in the first ten years (Olesen 1998). In the early 2000s, the total amount of infected farms was less than 30, all situated in a certain area of rivers with outlets in a brackish water lake on the west coast of Jutland. In 2009, fish farmers and their association endorsed a highly coordinated compulsory eradication programme to get rid of the disease for good. This plan included eradication of all positive farming localities and keeping some high-risk farms empty of fish for two years (brackish water farms) and some for six weeks every spring during a two-year period. The costs of the programme were partly financed by the European Fisheries Fund (EFF), which only compensated the value of the fish and the cost of their removal. Farmers paid the cost of cleaning and disinfecting the farm and bore the loss of income during the fallow. After 50 years of costly and laborious efforts, Denmark was declared VHS-free in 2013 (Olesen et al. 2013).

Other examples of successful eradications are from the UK and Norway. In the UK VHSV infection was detected for the first time in a rainbow trout farm situated in a river system on the east coast, in 2008 (Stone et al. 2008). The disease was eradicated from the farm, and no further spread was noticed in the epidemiological investigation of contacts or other farms in the same river system. The source of the infection was not clear, but one suspicion was raised concerning a fish smokery/processing site upstream that handled imported rainbow trout; infective material may have been released into the river. The isolated VHSV strain was genotype Ia, closely related to freshwater types in continental Europe and highly pathogenic for rainbow trout (Stone et al. 2008).

In Norway, VHSV was isolated from sea-reared rainbow trout with elevated mortality and abnormal swimming behaviour in 2007. The isolated agent was a marine genotype III, a group of strains that had earlier been demonstrated in wild fish in the North Sea but never from rainbow trout. It was known that earlier isolated marine strains, including genotype III, had been almost apathogenic to rainbow trout in infection trials (Skall et al. 2004b). In 2007, VHSV was also detected in an immunohistochemical staining from lesions on the internal organs of the diseased rainbow trout, confirming that this was a clinical VHS outbreak. The isolated VHSV strain was clearly pathogenic for rainbow trout in infection trials. It caused 70% cumulative mortality in an immersion trial and almost 100% by intraperitoneal injections (Dale et al. 2009). The infection was diagnosed in three farming localities in 2007, two new localities in 2008 and one in 2009 (National Veterinary Institute
Heavy eradication procedures were conducted with successful outcomes. No new isolations of VHSV have been made in the area after 2009 (Moldal, 2019).

Examples of VHSV epidemics where the outcomes of eradication procedures were not successful are from Sweden and from the Åland islands in Finland. In 1998, VHSV genotype Ib was detected in diseased sea-reared rainbow trout on the west coast of Sweden. Stamping-out procedures were performed but the infection reoccurred in 2000. VHSV was isolated from herring caught close to rainbow trout farms. The herring isolates were almost identical to the genotype that was isolated from the farmed rainbow trout. It was assumed that the herring during their spawning migration brought the infection repeatedly to the farm and made rainbow trout farming in that area impossible (Nordblom and Norell 2000, Jansson and Vennerström 2014).

Another area where the same genotypes of VHSV have been reported in both wild and farmed fish is British Columbia in Canada (Garver et al. 2013). VHSV genotype IVa has been reported repeatedly in farmed Atlantic salmon since the first isolation in 1995. These isolates have been compared genetically to isolates from wild marine fish species and found to be identical or nearly identical (Garver et al. 2013).

In Finland, VHS was first isolated at a brackish water farm producing rainbow trout in the Province of Åland in May 2000 and a few weeks later at a similar farm at Pyhtää on the south coast, approximately 330 km from the first location (Raja-Halli et al. 2006). The infection spread rapidly to several other farms in both areas (Figure 7). All fish in positive farms in Pyhtää were eradicated during 2001 and new cases of VHS have not occurred in that area since then. On the other hand, in the Province of Åland the disease continued spreading despite eradication measures performed in the farms, which were found positive first. In 2001, eradication measures in Åland were withdrawn as unsuccessful and too expensive. The area was classified as a VHSV-restriction area and no live or un gutted fish or farming equipment could be moved from this area.

In 2003, a third VHS outbreak occurred in a rainbow trout farm on the west coast of Finland in Pyhämäa. Stamping-out was performed and VHSV infection was not detected until 2008, when VHSV was detected in clinically healthy fish from screening samples taken in connection with the processing of the fish.

There was no known contact between the two fish farms in Åland and Pyhtää, where the first VHS outbreaks were found in 2000, and the strongest suspicion of the source of infection was wild herring. The diseased fish had not been fed wild herring, but both farms were situated close to harbours for herring trawlers, so there was close contact with trawled herring. The source of the first VHS virus infections in Finland is still open, but it is possible that marine VHSV mutated, or a new strain occurred in Finland. Einer-Jensen et al. (2004) reported in their evolutionary study that the Finnish isolates from rainbow trout resemble the old Danish isolates. They suggest that the Finnish isolate could have evolved from marine VHSV types similarly to what is believed to have happened in Denmark decades ago.

The farm in the third VHS-positive area (Pyhämäa) was known to have had contact with a fish farm in the VHSV-restriction area in the Province of Åland in 2003 and that was the most probable source of the first infection. The source of the second infection in 2008 was not clear. One possible reason could have been a massive escape earlier the same year of a
VHSV-infected rainbow trout population from a farm situated in the part of the restriction area in the Province of Åland that lies approximately 50 km from Pyhämaa (author’s own observations). All the studied VHSV isolates from rainbow trout in these three different areas in Finland have been of the same genotype II (99.3-100% nucleotide identity); wild fish e.g. herring and lamprey in the sea area surrounding the restricted areas have all been genotype II (Raja-Halli et al. 2006, Gadd et al. 2010, 2011). There are no reports of genotype II being isolated from wild fish.

The three VHS-restriction areas in Finland received an EU-approved VHS-eradication programme in 2003 (EC 2003). Two of the three restriction areas were declared VHSV-free after an intensified screening period according to the demands set by the European Commission, Pyhtää in 2005 and Pyhämaa in 2011 (Figure 7). Eradication measures in Åland have not been successful. Reinfections have occurred, although some farms have been emptied of fish, all equipment disinfected, and the farms kept empty for several months before new fingerlings were transferred from the disease-free area. These setbacks reduced the eagerness of farmers to participate in eradicating the disease from Åland. The appearance of VHS in the magnitude present back in the early 2000s was considered a significant threat to the disease-free status of the continental area (Lyytikäinen et al. 2007).

Strict biosecurity on fish farms and effective disease monitoring to detect infections as soon as possible is important in the control of most fish diseases, including VHS. In Norway, routine clinical inspections performed by authorized veterinarians and fish health biologists has proven to be a good tool in surveillance for freedom from VHS in marine farmed salmonids (Lyngstad et al. 2016). If infection occurs, movement of fish material should be restricted, as infected fish or eggs are the main carries for spreading the disease (Wolf 1988, Oidtmann et al. 2011a, 2011b, Reichert et al. 2013, Bang-Jensen et al. 2014). Un-disinfected farming equipment, boats and personnel may also spread the disease to uninfected farms (Bovo et al. 2005a). In addition, infection is spread via outlet water and escapees from infected farms, and via birds (Olesen and Vestergård Jorgensen 1982).

No effective vaccine is on the market and the most effective control is still avoidance. DNA vaccines against VHS seem to be the most promising (Dalmo 2018).
Figure 7. Amount of VHS cases in the three restriction areas A (Åland), B (Pyhtää) and C (Pyhämaa) regarding VHS during 2000-2019. *Free since 2008 **Free since 2011
3. Aims of the study

The overall aim of the study was to gain knowledge of the epidemiological factors needed for VHS management in VHSV-positive brackish water fish farms farming rainbow trout in net pens in Finland. The specific objectives were as follows:

1. To test the ability of different surveillance procedures and diagnostic methods to find VHSV-infected populations (I)
2. To test if wild fish living in the close vicinity of VHSV-positive rainbow trout populations are carriers of VHSV (II)
3. To study if blue mussels could be carriers of VHSV (III)
4. To study if VHSV can be found in the environment where VHSV-positive populations have been farmed or are handled for processing (III)
4. Materials and methods

Samples that were included and tested in this study were collected from wild fish, farmed fish, infection trials using wild fish, farmed whitefish and wild blue mussels. Samples were also collected from the environment of fish farms with VHS-infected rainbow trout populations and from processing plants handling VHSV-infected fish. This study received ethics permission for sampling and testing fish dno. ESLH-2006-08289/Ym23. A summary of the design of the different studies performed is presented in table 1.

Table 1 Design of different studies performed

<table>
<thead>
<tr>
<th>Publication</th>
<th>Description of study</th>
<th>Study type</th>
<th>Sampling frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Syndromic surveillance, farmed rainbow trout</td>
<td>Field study</td>
<td>Always if any sign of disease</td>
</tr>
<tr>
<td>I</td>
<td>Active random, farmed rainbow trout</td>
<td>Field study</td>
<td>Once every spring and autumn, from arrival at the farm until slaughter</td>
</tr>
<tr>
<td>I</td>
<td>Active non-random, farmed rainbow trout</td>
<td>Field study</td>
<td>Once every spring and autumn, from the first VHSV infection noticed until slaughter</td>
</tr>
<tr>
<td>I</td>
<td>EU reference, farmed rainbow trout</td>
<td>Field study</td>
<td>Once a year in uninfected farms; every second year in infected farms</td>
</tr>
<tr>
<td>II</td>
<td>Screening of wild fish in VHSV-positive farms</td>
<td>Field study</td>
<td>Every spring and autumn during the study period of 4 years</td>
</tr>
<tr>
<td>II</td>
<td>Infection trial with wild fish</td>
<td>Field study</td>
<td>Days 0, 10, 14, 21 and 35 post-infection</td>
</tr>
<tr>
<td>II</td>
<td>Infection trial with farmed whitefish</td>
<td>Field study</td>
<td>Days 0, 10, 14, 21 and 35 post-infection</td>
</tr>
<tr>
<td>III</td>
<td>Screening of wild blue mussels in VHSV-positive farms</td>
<td>Field study</td>
<td>At least once every spring and autumn for two years</td>
</tr>
<tr>
<td>III</td>
<td>Infection trial with blue mussels using cultured virus</td>
<td>In vitro</td>
<td>Days 0, 1, 2, 3 and 6 post-infection</td>
</tr>
<tr>
<td>III</td>
<td>Infection trial with blue mussels using VHSV-infected rainbow trout</td>
<td>Field study/In vitro</td>
<td>Days 0, 1, 2, 3, 4, 6, 8, 11, 14, 22, 27 and 29 post-infection</td>
</tr>
<tr>
<td>III</td>
<td>Testing of sea water for VHSV</td>
<td>Field study</td>
<td>Springtime and wintertime at VHSV-positive fish farms</td>
</tr>
<tr>
<td>III</td>
<td>Testing of wastewater for VHSV in a processing plant</td>
<td>Field study</td>
<td>Twice at a plant processing fish from VHSV-positive fish farms</td>
</tr>
</tbody>
</table>
4.1 Description of the study area (I, II, III)

The study was performed on VHS-positive fish farms farming rainbow trout for consumption in net pens, and processing plants handling VHSV-infected fish populations in an area that has been placed under restrictions regarding VHS since 2000. Another farm situated outside the restriction area, but in similar conditions on the coast of the mainland, was used as a control farm in the studies reported in article II. The fish farms in the study areas received their fry from Finnish inland farms that use natural water from lakes and rivers or well water. Fish were also transported from farms situated on the coastal area outside the VHS-restriction area. VHS has never been reported in the inland area or the earlier-mentioned coastal area where fish farming has been continuously screened for fish disease including viral diseases since 1969. Fry had also been imported to the study area, including farm A of this study, from officially VHS-free farms in Denmark before and during this study.

The net pens in the study areas are anchored to the bottom or to the nearby shore. The study area consists of several small islands. The fish farms are scattered around them and can mostly be reached only by boat. The environmental conditions set demands for fish farming in the study area. Fish farming is performed in separate localities during summer and winter. Winter localities are often located in shallow bays and by surrounding islands close to the mainland. Therefrom they are easily reached and protected from harsh weather conditions in autumn and especially during winter when the sea may get an ice cover.

During summer, water temperatures often rise over 20 °C in the winter localities and fish are moved to the summer localities situated offshore with the cooler and deeper waters more optimal for farming salmonids. The farming licences restrict the amount of fish that may be placed in winter localities and order that fish must be moved to summer localities before a certain date. Fish that have achieved slaughter size in autumn, are often moved as close to the processing plants as possible or are transported by well boats directly from the summer localities for processing. Fish that will not attain slaughter size until the next year are often kept in winter farming localities for one year and then transported to a summer locality. These winter localities are also often situated next to the processing plant. The processing plant is also the main storage for fish food and farming equipment. The dock of the processing is the home port for the boats that are used for the daily servicing of the farming localities.

The fish farms in the study area have a high number of different wild fish species living in the close vicinity of the net pens. The presence of the different fish species depends on their migrating behaviour: some are long-distance ‘travellers’ like sea trout and herring, whereas perch only migrate to nearby shore or bays.

At the beginning of the study there were five fish farming companies in the VHS-restriction area, each operating on several farms or farming localities. Most of the fish farms in the restriction area had experienced an outbreak of VHS in their fish populations before this study was performed.
4.2 Screening of farmed fish populations, wild fish and blue mussels for the presence of VHSV (I, II, III)

Fish populations from the fish farms of two enterprises in the restriction area, later called farm A and farm B, were screened for the presence of VHSV using four programmes (table 2) (I). The first programme was based on syndromic surveillance, where the farmers were asked to submit 5-10 affected fish to the laboratory for autopsy and diagnostics every time they noticed any signs of disease in their fish populations. The second programme was an active targeted surveillance where the presence of VHSV was tested from three rainbow trout populations, one from farm A and two from farm B. In the third programme, screening of VHSV was started in two rainbow trout populations after a VHS outbreak was reported and additionally two other populations of rainbow trout from the same locality where signs of VHS were not noticed. The fourth programme was the official screening programme of the VHS-restriction area. Serum samples to detect antibodies against VHSV were collected from the fish sampled in programmes 2 and 3.

Wild fish were caught using special designed nets with four different mesh sizes to be tested for VHSV during a period of four years in the VHS-restriction area (II). The fish were caught in the close vicinity of the fish farms of enterprises A and B with VHSV-positive fish populations. Blue mussels were collected from the anchor ropes of the net pens and the surface of the net pens of fish farms with VHSV-positive fish populations (III).

4.3 Infection trials

4.3.1 Wild fish and farmed whitefish (II)

Wild perch and roach were caught with bow nets in the coastal area outside the VHS-restriction area to be used in a challenge test at a VHSV-positive fish farm. The live-caught fish were transported in aerated tanks to the VHS-positive fish farm A and placed into small net pens to be tested if they were infected by VHSV from rainbow trout experiencing a clinical outbreak of VHS (Figure 8). The wild fish were sampled for VHSV testing before transport to the restriction area. Likewise, farmed whitefish were transported from the VHS-free inland area to test if they could be infected by VHSV. Control groups of all three fish species were placed in similar cages and conditions at control farm C situated outside the VHS-restriction area.
4.3.2 Blue mussels (III)

Mussels for two different bath challenges were collected from the anchor ropes of control farm C and transported in cooled boxes on wet paper to be used in two bath challenges with VHSV (Figure 9). The collected mussels were sampled for the presence of VHSV before the challenge started.

In the first bath challenge, two groups (group I and II) of 60 blue mussels each were placed into separate aerated aquariums with sea water collected from control farm C. The trial was performed in the cool room of the autopsy facilities at the Finnish Food Safety Authority (today the Finnish Food Authority) (Figure 10). The mussels were exposed to a suspension of VHSV strain Fika422, genotype Id (GenBank accession no. AY546615), 5 ml each, collected from a cell culture that was added to the aquarium water of both test groups I and II. Group I was exposed for 6 h and group II for 1 day. The third aquarium functioned as a control group and was not exposed to VHSV. At the end of the exposure, the aquarium water of all three aquariums was changed and clean sea water was added. The vividness (actively filtrating water and closing shell by physical touch) of the blue mussels was confirmed before every sampling and change of water, to ensure that the mussels were filtrating water. The mussels were screened for 6 days.

The second bath challenge was performed by placing four rainbow trout infected by VHSV in two aerated aquariums containing 100 mussels each. Two rainbow trout were placed in each aquarium. The exposure lasted for 10 min in group I and 20 min in group II thereafter the rainbow trout were removed from the bath challenge, euthanised, autopsied and their organ samples collected for testing of VHSV individually. The bath challenge of the mussels continued for 4 h after the rainbow trout had been removed. After the challenge, the mussels were transported in a cooled box on moist paper to the facilities where the first
challenge trial was performed. The challenged blue mussels were placed in two aquariums, treated and sampled as in the first bath challenge, but the follow-up continued for 29 days. The second trial also included a control group, as in the first trial. The sampling scheme for both bath challenges is described in table 5.

Figure 9. Wild blue mussels were collected from the anchor ropes of the net pens that are covered by a thick layer of blue mussels. (Photo: Pia Vennerström)

Figure 10. Blue mussels were challenged in small aerated aquariums, a, b test aquariums and c control group. (Photo: Pia Vennerström)
4.4 Sampling and treatment of sea water, wastewater and sediment (III)

To test if VHSV could be detected from sea water close to rainbow trout populations experiencing a clinical outbreak of VHS, sea water was collected from the surface and from 2 m depth of fish farms A and B during a clinical outbreak of VHS. Sea water was also collected next to the loading dock of a processing plant processing VHSV-infected fish populations. Sediment was collected from the bottom of fish farm A with VHSV-positive fish populations.

To test if VHSV could be detected in wastewater from the processing line of a processing plant handling VHSV-positive fish populations, samples were collected from the wastewater of different parts of the processing line; CO₂ stunning basin, bleeding basin, kidney remover, drain before liquid waste disinfection and drain after disinfection.

Water samples were treated with methods described by Maunula et al. (2012) and tested at the University of Helsinki, Faculty of Veterinary Medicine, Department of Food Hygiene and Environmental Health. Water samples were prefiltered through a Waterra® filter FHT-700, Waterra (Anon 2000) from which virus particles were eluted using 50 ml of 50 mM glycine-3% beef extract. The filtration continued through a GF/F membrane and virus eluted from this filter by 1 ml AVL lysis buffer.

Aquarium water from the infection trials and the wastewater samples was not filtered before RNA extraction. Eluates from both filters and unfiltered aquarium water were subjected to RNA extraction by viral RNA Mini Kit (Qiagen).

Sediment samples were diluted by taking 5 g of each sample and adding 1 mL of PBS. RNA extraction from the diluted and stirred sediment samples was performed using a NucliSens magnetic extraction kit (Biomérieux, Boxtel, Netherlands).

4.5 Diagnosing VHSV

4.5.1 Virus detection by virus isolation and qRT-PCR (I, II, III)

The intention of this study was not to find new diagnostic methods, but to use methods that had already been tested and found to be useful and reliable. Virus isolation in cell cultures is considered the gold-standard method for detecting VHSV and was used in this study for all samples except some samples taken from sea water and wastewater. Virus isolation is laborious and time-consuming and therefore we tested three molecular methods, conventional RT-PCR, nested PCR and qRT-PCR, to find a method suitable for detecting VHSV in this study. A qRT-PCR method earlier described by Chico et al. (2006) with minor modifications was chosen to be tested as the most appropriate PCR method. qRT-PCR was used in parallel with cell culture from the same suspensions used for cell culture except for some of the water and wastewater samples that were only tested by qRT-PCR (I, II, III).
**Virus isolation in cell culture (I, II, III)**

Tissue samples of the brain, anterior kidney and spleen from fish and hepatopancreas from blue mussels were processed according to standard virological procedures (EC 2006, EC 2015, OIE 2019). Organ samples of a maximum of 5 fish or blue mussels were pooled, homogenised and centrifuged (4000 × g, 15 min). Samples from sea water, aquarium water from infection trials and wastewater were only stirred before centrifuging. The supernatant was collected for immediate inoculation into 24-well tissue culture plates (Nunc) with monolayer cell cultures of bluegill fry fibroblasts (BF-2) or epithelioma papulosum cyprinid (EPC) epithelial cells (Olesen and Vestergård Jørgensen 1992). The samples were inoculated in BF-2 and EPC cells no later than 24 h post-euthanasia except for programme 4 (official screening in article I), where the instructions given in Commission Decision 2001/183/EC (EC 2001) were followed. The maximum time between euthanasia of the fish and inoculation of samples into cell culture was 48 h in the last-mentioned method.

**Real-time RT-PCR for examining the presence of VHSV from tissue suspensions, cell culture, sea water, aquarium water, wastewater and sediment (I-III)**

A volume of 1 ml of the same organ suspension and water samples that were used for virus isolation was frozen at −80°C for real-time RT-PCR. RNA extraction was carried out using RNeasy Mini Kit (Qiagen) starting with 200 µl suspension according to the manufacturer’s protocol, and the final elution volume was 32 µl.

RT-PCR was performed using QuantiTect Probe RT-PCR Kit (Qiagen) according to the manufacturer’s instructions. Five microliters of extracted RNA was used in a 25 µl reaction volume. The final concentrations of the primers and the probe were 300 and 100 nM, respectively. The RT reaction profile was: 30 min at 50°C, 15 min at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C.

The primers and the probe for the real-time RT-PCR were manufactured (MedProbe) according to the VHSV sequence from GenBank accession no. D00687 after Chico et al. (2006). The probe was 5-end labelled with FAM fluorescent dye and 3-end labelled with TAMRA fluorescent dye.

To test the sensitivity and repeatability of the qRT-PCR method used, we made a dilution series of VHSV strain Fi-ka422, AY546615 (Einer-Jensen et al. 2004). The dilution series was run several times to test the repeatability of the test and to estimate the threshold cycle (Ct) cut-off (Figure 11). The virus titre was estimated using Spearman-Kärber’s method (Mahy and Kangro 1996), estimating 50% tissue culture infective dose (TCID50/ml).
Figure 11. A: The qRT-PCR curves of 10-fold dilutions ($10^{-2}$-$10^{-8}$) of VHSV strain Fi-Ka422, AY546615. B: The threshold cycle of repeated qRT-PCR of dilution series of Fi-Ka422.

4.5.2 Strand specific qRT-PCR (II, III)

The sea water of the fish farms with VHSV-infected rainbow trout was assumed to be highly contaminated by VHSV during the study. Several of the sampling occasions were even performed during clinical outbreaks of VHS, and a way to prove that the positively tested
tissue samples were a result of virus infection and not contamination was needed. For this purpose, a new strand-specific qRT-PCR method was developed to test for negative- and positive-stranded VHSV products formed during VHSV replication in fish cells. This strand-specific qRT-PCR method is based on a method previously described by Purcell et al. (2006), who used it to test for replication products of infectious haematopoietic virus (IHNV) in salmon.

Controls for the strand-specific method were created with in vitro transcription from cloned VHSV N gene amplicons. To create a template for positive and negative control RNA, an 810 bp amplicon from VHSV N gene PCR was inserted into a pSC-A plasmid and transfected into StrataClone SoloPack competent cells (StrataClone™ PCR Cloning Kit, Stratagene) according to the manufacturer’s instructions. The resulting plasmids were purified using QIAprep Spin Mini – prep Kit (Qiagen) and verified by restriction digestion and sequencing with the universal T3 and T7 primers. Sequencing was performed at the Institute of Biotechnology, University of Helsinki, Finland.

The control RNA for strand-specific qRT-PCR was prepared in 2 separate in vitro transcription reactions to produce both positive- and negative-strand RNA. Based on sequencing, the orientation of the insert could be determined, and linearization of the plasmid was performed with BamHI (Fermentas) or HindIII (Fermentas) for positive- or negative-strand RNA transcription, respectively. In vitro transcription of linearized plasmids was performed using MAXIscript T7/T3 Transcription Kit (Ambion, Applied Biosystems) according to the manufacturer’s instructions. Both the positive- and negative-strand RNA concentrations were adjusted to 200 ng μl⁻¹, and the RNA was aliquoted and stored at −70°C.

For the detection of positive-stranded RNA, 0.5−2 μg of total RNA was isolated from fish organ pools using RNasy Mini Kit. Reverse transcription was performed according to Purcell et al. (2006) and Chico et al. (2006). Tagged antisense primer (reverse) was used to synthesise positive-strand based cDNA and tagged sense primer (forward) to synthesise negative-strand based cDNA (Vennerström et al. 2018).

### 4.5.3 Serology (I)

Serum samples from rainbow trout from farms with VHSV-infected populations were tested for antibodies against VHSV. Serology may reveal VHSV-infected fish populations that have been missed in screening for the virus.

The collected blood samples were centrifuged (3000 × g, 15 min) to obtain serum. The serum samples were heat-inactivated for 30 min at 45°C (Olesen et al. 1991) and frozen (−80°C) until examination. The serum samples were tested for the presence of VHSV antibodies using an indirect ELISA method (diagnostic specificity, Sp: 1.0; diagnostic sensitivity, Se: 0.92) (Olesen et al. 1991). The ELISA results of this study were verified by sending a set of samples to be tested in parallel in another laboratory with experience of VHSV serology. This was performed because there have been reports that the results may depend on the used virus strain (antigen) and unspecific factors in the tested serum itself (Fregrenda-Grandes and Olesen 2007). The laboratory was in France at
Ploufragan/Plouzané, Unité de pathologie virale des poissons (Afssa). The samples were tested with the same ELISA method and with a serum neutralisation test (diagnostic specificity Sp 1.0 and sensitivity Se 0.6) (Olesen and Vestergård Jørgensen 1986, Olesen et al. 1991, Castric et al. 2009). The received results were consistent with our results.

4.6 Statistical analyses (I, II, III)

To test objective 1 of this study, the effectiveness of different surveillance programmes in detecting VHSV infections was estimated using a binomial generalised linear model (GLM) (logit link): Logit(Y ) = a(Programme1) + b(Programme2) + c(Programme3) + d(Programme4) + eT + fT^2 where Y = a positive detection of VHSV, T = temperature (°C) and a, b, c, d, e and f = coefficients. Programme 4 was treated as a reference category and was the intercept of the estimated model. An omni-bus test was used to determine whether the model was better than the intercept-only model. A model without temperature as a covariate was estimated to assess whether the inclusion of temperature changed the relative efficiencies of the programmes. Probabilities of detection were calculated from a logistic model in the usual way: Probability of detection = e(relevant part of the GLM)/[1+e(relevant part of the GLM)]. For more information, see Dohoo et al. (2009). Statistical analyses were performed using IBM SPSS Statistics version 22.

The modified qRT-PCR test was compared with virus isolation by cell culture (gold-standard test for detecting VHSV) in order to calculate the diagnostic Se and Sp using EpiTools (Sergeant 2016). The threshold cycle (Ct) cut-off was estimated using 2-graph receiver operating characteristic (TG-ROC) curves (Caraguel et al. 2011) with EpiTools (Sergeant 2016).

In objective 2 of this study, the 95% confidence intervals for observed proportions between different wild fish species were calculated using EpiTools (Sergeant 2016) using Jeffrey’s method (Brown et al. 2001). The median prevalence estimate (%) was calculated using R (R Core Team 2016).

Objectives 3 and 4 were descriptive studies where the 95% confidence intervals (CI) for percentages were calculated similarly as for objective 2 using Wilson’s method.
5. Results

5.1 Surveillance programmes and diagnostic methods (I)

The sampling scheme of the different surveillance programmes are presented in table 2 and results in figure 12. VHSV was detected by virus isolation in 75% of the 12 sampling occasions, when tested from fish showing signs of disease that were sent in for autopsy by fish farmers (programme 1). This procedure gave up to a 17 times higher probability of detection of VHSV than the official screening procedure (programme 4). Programme 2, designed as the official programme 4 but including more frequent sampling of the same fish population than the official programme, gave a result that did not differ from the official programme 4 (Generalised Linear Model, GLM, P>0.05). When screening was performed in fish populations that were known to have been VHSV-positive when screening started, the probability of detecting VHSV was 7.7-8.3 times higher than when the official programme 4 was used (GLM, P<0.05).

Antibodies against VHSV were detected in only 4 serum samples out of 120 in programme 2. In programme 3, where the tested populations had experienced a clinical outbreak just before the start of the surveillance, all populations were positive on several occasions (I, Table 2).

qRT-PCR, with cut-off set at Ct value of 36, corresponded well with the virus isolation on the separate sampling occasions in programmes 1-3 (kappa value 0.877). The diagnostic sensitivity and specificity of qRT-PCR was 1 and 0.959 respectively when using virus isolation in cell culture as the gold-standard test for detecting VHSV.

| Table 2 Code of action in different surveillance programmes. N number, qRT-PCR real-time reverse transcriptase PCR. |
|---|---|---|---|
| **Action** | **Program 1** | **Program 2** | **Program 3** | **Program 4** |
| Passive | Active/random | Active non-random | EU-reference |
| **Sampling times** | always if mortality elevated or signs of disease present | once every spring and autumn | once every spring and autumn | once a year in not infected farm ever 2nd year in infected farm |
| **N of fish sampled/sampling time** | 1-10 | 30 | 30 | 30 |
| **Sampling from diseased fish** | always | if noticed | if noticed | if noticed |
| **N of pools for virus isolation/sampling time** | 1-5 | 6 | 6 | 3 |
| **N of pools tested by qRT-PCR/sampling time** | 1-5 | 6 | 6 | 3 |
| **N of serum samples/sampling time** | 0 | 30 | 30 | 0 |
| **Temperature** | <15 °C | <15 °C | <15 °C | <15 °C |
5.2 Wild fish and whitefish (II)

VHSV was not detected in any of the 1,636 wild fish, representing 17 different fish species, that were caught and tested for VHSV in the vicinity of infected fish farms. Four pooled samples of four fish species (ruffe Gymnocephalus cernuus, herring Clupea harengus membras, rainbow trout Oncorhynchus mykiss and four horn sculpin Triglopsis quadricornis) gave a weak signal (Ct >36) with qRT-PCR (Table 3). These four samples tested negative with the strand-specific RT-PCRs, indicating that VHSV replication had not occurred in the tested samples.

The wild perch and roach that were exposed to VHSV at a fish farm with rainbow trout experiencing a clinical outbreak did not test positive during the infection trial. On the contrary, whitefish which were also exposed in the same way as perch and roach tested positive for VHSV. One of the three parallel groups of exposed whitefish was positive with both virus isolation and qRT-PCR. In addition, the strand-specific RT-PCR method, testing for positive-stranded RNA products that are present in a fish cell only during virus replication also gave a positive result. The isolated VHSV strains from the challenged whitefish and diseased rainbow trout of the fish farm, where the challenge was performed, and from other VHS-positive fish farms during 2001 and 2004 were sequenced and found to be almost identical; nucleotide identity was 99.4-99.9% (Figure 13). All the control fish in the control farm outside the restriction area tested negative for VHSV.

A Sprivivirus was isolated from all fish species in the challenge test. Spriviviruses grow in the same cell cultures as those used for VHSV isolation and were confirmed in a study performed by Holopainen et al. (2017).
Table 3 Amount of wild fish tested for VHSV at farms with VHSV positive rainbow trout during 2005-2008. VHSV could not be isolated from these fish species. *Four species gave a weak positive (C(t) > 36) reaction, in one organ pool per species, by qRT-PCR: real-time reverse transcription PCR, but tested negative with the positive strand specific qRT-PCR.

<table>
<thead>
<tr>
<th>Species</th>
<th>Autumn</th>
<th>Spring</th>
<th>Sum.</th>
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</thead>
<tbody>
<tr>
<td>Perch (Perca fluviatilis)</td>
<td>300</td>
<td>213</td>
<td>513</td>
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<tr>
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<td>Roach (Rutilus rutilus)</td>
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<td>Three-spine stickleback</td>
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<td>Ruffe (Gymnocephalus cernuus)</td>
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<td>Herring (Clupea harengus membras L.)</td>
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</tr>
<tr>
<td>Rudd (Scardinius erythrophthalmus)</td>
<td>4</td>
<td>16</td>
<td>20</td>
</tr>
<tr>
<td>Rainbow trout (Oncorhynchus mykiss)</td>
<td>1</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Four horned sculpin</td>
<td>2</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Flounder (Platichthys flesus)</td>
<td>1</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Sea trout (Salmo trutta)</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Straight nosed pipefish</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Ealpout (Zoarces viviparus)</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Ide (Leuciscus idus)</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Pike (Esox lucius)</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>sum.</td>
<td>611</td>
<td>1025</td>
<td>1636</td>
</tr>
</tbody>
</table>
Figure 13 Phylogenetic analysis of Finnish VHSV- strains isolated from white fish (Fi08.22WF, Fi08.23WF, Fi08.24WF) and rainbow trout (Fi08.50RT, Fi06.59RT, Fi06.108RT, FiA02a.01, FiA19.04) together with selected strains of VHSV belonging to genotypes I-IV. Tree is based on complete coding sequence of the glycoprotein (G) gene (1524 nt), and it was generated by using the neighbour-joining method in MEGA 4.1 software (Tamura et al. 2007). The reliability of the tree was determined by 1000 dataset bootstrap resampling; numbers on the tree represent percentage of bootstrap support. The scale bar indicates nucleotide substitutions per site. (Vennerström et al. 2018, Supplement)

5.3 Blue mussels (III)

VHSV was not detected in any of the 62 pools of 193 wild blue mussels tested (CI 0.00-0.06), except for one pool that gave a weak signal Ct> 36 by qRT-PCR in May 2006 from samples from the infected fish farm. The results are presented in table 4.

According to the two bath challenge trials performed in this study, VHSV does not replicate in blue mussels exposed to live VHSV (Table 5).

In the first trial using virus suspension, VHSV was only isolated from the cell culture of the organ suspension of mussels from group I at the end of the 6 h exposure. All organ
suspensions of mussels from group II, exposed to the virus for 1 d, were negative by virus isolation (CI 0.05-0.23). qRT-PCR gave positive signals for VHSV RNA in both test groups I and II throughout the whole trial of 6 days.

In the second infection trial, blue mussels were exposed to VHSV by keeping them in an aquarium with VHSV-infected rainbow trout. The virus could not be isolated from the exposed blue mussels at any time of the trial (CI 0.00-0.07). VHSV RNA was detected in the organ suspensions of the mussels for 3 days, but weak signals (Ct>36) were detected throughout the whole 29-day trial. In the second trial, samples from the aquarium water were also collected and tested by virus isolation and qRT-PCR for VHSV RNA. VHSV could not be isolated from the aquarium water at any time, but VHSV RNA was detected in the aquarium water at the time when the rainbow trout were removed from the bath challenge.

Table 4. Results of testing for viral haemorrhagic septicaemia virus (VHSV) in the hepatopancreas of wild blue mussels from two VHS-positive fish farms (A and B) farming rainbow trout for consumption in the Province of Åland and from a similar farm situated in a VHS-free zone on the west coast of continental Finland used as a control (C). N = number; Nd = not done; RT-PCR = reverse transcriptase polymerase chain reaction. a weak signal with threshold cycle cut-off >36 (Vennerström et al. 2020).

<table>
<thead>
<tr>
<th>Farm / Time of sampling</th>
<th>N mussels</th>
<th>N pools</th>
<th>Cell culture</th>
<th>Real-time RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Company 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A / April 2006</td>
<td>13</td>
<td>7</td>
<td>0/7</td>
<td>Nd</td>
</tr>
<tr>
<td>A / May 2006</td>
<td>10</td>
<td>10</td>
<td>0/10</td>
<td>1ª/10</td>
</tr>
<tr>
<td>A / November 2006</td>
<td>100</td>
<td>20</td>
<td>0/20</td>
<td>0/20</td>
</tr>
<tr>
<td><strong>Company 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B / May 2006</td>
<td>10</td>
<td>5</td>
<td>0/5</td>
<td>Nd</td>
</tr>
<tr>
<td>B / June 2006</td>
<td>50</td>
<td>10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td><strong>Control farm</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C / May 2007</td>
<td>10</td>
<td>10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>193</td>
<td>62</td>
<td>0/62</td>
<td>1ª/50</td>
</tr>
</tbody>
</table>
Table 5. Viral haemorrhagic septicaemia virus (VHSV) isolations and real-time reverse transcriptase polymerase chain reaction (RT-PCR) results from two bath challenges of blue mussels with VHSV grown in cell culture and VHSV from infected rainbow trout. In both trials, Groups I and II are test groups and group III is a negative control group of which all results were negative and are not shown in the table. \( d = \) days; \( h = \) hours; \( N = \) number; \( Nd = \) not done; RT-PCR = reverse transcriptase polymerase chain reaction. \( a \) weak signal with threshold cycle cut-off >36. (Vennerström et al. 2020)

<table>
<thead>
<tr>
<th>Time of sampling</th>
<th>Group</th>
<th>Virus isolation in cell culture</th>
<th>Real-time RT-PCR</th>
<th>Real-time RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bath challenge with VHSV</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (before challenge)</td>
<td>I, II, III</td>
<td>0/5</td>
<td>Nd</td>
<td>Nd</td>
</tr>
<tr>
<td>6h (at end of challenge)</td>
<td>I</td>
<td>5/5</td>
<td>5/5</td>
<td>Nd</td>
</tr>
<tr>
<td>1d (at end of challenge)</td>
<td>I</td>
<td>0/5</td>
<td>3/5</td>
<td>Nd</td>
</tr>
<tr>
<td>1d (at end of challenge)</td>
<td>II</td>
<td>0/5</td>
<td>4/5</td>
<td>Nd</td>
</tr>
<tr>
<td>2d</td>
<td>I</td>
<td>0/5</td>
<td>1/5</td>
<td>Nd</td>
</tr>
<tr>
<td>2d</td>
<td>II</td>
<td>0/5</td>
<td>2/5</td>
<td>Nd</td>
</tr>
<tr>
<td>3d</td>
<td>I</td>
<td>0/5</td>
<td>0/5</td>
<td>Nd</td>
</tr>
<tr>
<td>3d</td>
<td>II</td>
<td>0/5</td>
<td>4/5</td>
<td>Nd</td>
</tr>
<tr>
<td>6d</td>
<td>I</td>
<td>0/5</td>
<td>3/5</td>
<td>Nd</td>
</tr>
<tr>
<td>6d</td>
<td>II</td>
<td>0/5</td>
<td>2/5</td>
<td>Nd</td>
</tr>
<tr>
<td><strong>Bath challenge with VHSV-infected rainbow trout</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (before challenge)</td>
<td>I, II, III</td>
<td>0/3</td>
<td>0/3</td>
<td>0/1</td>
</tr>
<tr>
<td>At end of 10-min challenge</td>
<td>I</td>
<td>0/2</td>
<td>2/2</td>
<td>2/2</td>
</tr>
<tr>
<td>At end of 20-min challenge</td>
<td>II</td>
<td>0/2</td>
<td>1/2</td>
<td>2/2</td>
</tr>
<tr>
<td>1d</td>
<td>I</td>
<td>0/2</td>
<td>1/2</td>
<td>1(^a)/1</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>0/2</td>
<td>1(^a)/2</td>
<td>1(^a)/1</td>
</tr>
<tr>
<td>2d</td>
<td>I</td>
<td>0/2</td>
<td>2/2</td>
<td>1(^a)/1</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>0/2</td>
<td>0/2</td>
<td>1(^a)/1</td>
</tr>
<tr>
<td>3d</td>
<td>I</td>
<td>0/2</td>
<td>1/2</td>
<td>0/1</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>0/2</td>
<td>0/2</td>
<td>0/1</td>
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<tr>
<td>4d</td>
<td>I</td>
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<td>0/1</td>
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<td>II</td>
<td>0/2</td>
<td>0/2</td>
<td>0/1</td>
</tr>
<tr>
<td>6d</td>
<td>I</td>
<td>0/2</td>
<td>1(^a)/2</td>
<td>0/1</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>0/2</td>
<td>1(^a)/2</td>
<td>0/1</td>
</tr>
<tr>
<td>8d</td>
<td>I</td>
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<td>0/2</td>
<td>0/1</td>
</tr>
<tr>
<td></td>
<td>II</td>
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<td>0/2</td>
<td>0/1</td>
</tr>
<tr>
<td>11d</td>
<td>I</td>
<td>0/2</td>
<td>0/2</td>
<td>0/1</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>0/2</td>
<td>0/2</td>
<td>0/1</td>
</tr>
<tr>
<td>14d</td>
<td>I</td>
<td>0/2</td>
<td>0/2</td>
<td>0/1</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>0/2</td>
<td>0/2</td>
<td>0/1</td>
</tr>
<tr>
<td>22d</td>
<td>I</td>
<td>0/2</td>
<td>1(^a)/2</td>
<td>0/1</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>0/2</td>
<td>0/2</td>
<td>0/1</td>
</tr>
<tr>
<td>27d</td>
<td>I</td>
<td>0/2</td>
<td>0/2</td>
<td>0/1</td>
</tr>
<tr>
<td></td>
<td>II</td>
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<td>0/2</td>
<td>0/1</td>
</tr>
<tr>
<td>29d</td>
<td>I</td>
<td>0/2</td>
<td>1(^a)/2</td>
<td>0/1</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>0/2</td>
<td>0/2</td>
<td>0/1</td>
</tr>
</tbody>
</table>

N samples positive / N samples tested

Hepatopancreas samples of mussels

Aquarium water

48
5.4 Sea water, wastewater and sediment (III)

The results of this study are presented in table 6. VHSV RNA was detected in one sample out of 40 filtered sea water samples collected in April–May 2008 from two fish farms with rainbow trout experiencing a clinical outbreak of VHS (CI 0.004-0.129). The positive sample was collected from the surface of a farm situated nearby a processing plant processing VHSV-positive rainbow trout. Virus isolation was not performed from these samples.

All four sea water samples collected in January and March 2009 were positive for VHSV RNA (CI 0.51-1.0). These samples were also filtered before testing by qRT-PCR. Virus isolations by cell culture performed from these samples were all negative.

All 10 sediment samples collected next to a net pen with clinically diseased rainbow trout were negative for VHSV RNA (CI 0.0-0.28).

All liquid waste samples collected from a processing plant processing VHSV-infected rainbow trout tested positive by qRT-PCR in January 2009 (CI 0.43-0.90). The positive samples were collected before the liquid waste was treated with disinfectants. VHSV was isolated by cell culture in 78% of the same samples. After the final disinfection of the liquid waste, no virus could be detected by either method.

In March 2009 the sampling from the processing plant was repeated, but only clinically healthy whitefish were processed at the processing plant. VHSV was isolated from 63% of the samples (CI 0.31-0.86). The liquid waste disinfection system was not running at the time of the second sampling, and therefore disinfected effluent could not be collected for testing.
Table 6. Results of testing for viral haemorrhagic septicaemia virus (VHSV) in sea water, sediment and liquid wastewater from two VHSV-positive fish farms and a plant processing VHSV-positive fish. CPE = cytopathic effect; N = number; Nd = not done; pos = VHS-positive samples; PP = processing plant of company 2; RT-PCR = reverse transcriptase polymerase chain reaction; x = water and liquid waste samples were filtered before testing with real-time RT-PCR (Vennerström et al. 2020).

<table>
<thead>
<tr>
<th>Farm / time of sampling / water temperature</th>
<th>Type of sample / Origin of sample</th>
<th>N samples (pooled for direct real-time RT-PCR)</th>
<th>Water filtering</th>
<th>Virus isolation N CPE pos / N samples</th>
<th>Real-time RT-PCR N pos / N CPE pos cell culture</th>
<th>N water samples pos / N tested</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Company 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A / April–May 2008 / 4°C</td>
<td>Seawater / net pens with VHSV-positive trout</td>
<td>21 x Nd Nd 1/21</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A / April 2008 / 4°C</td>
<td>Sediment / under net pens with VHS-positive trout</td>
<td>10 Nd Nd 0/10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Company 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B / May 2008 / 10°C</td>
<td>Seawater / net pens with VHS-positive trout</td>
<td>19 x Nd Nd 0/19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PP / January 2009 / 2°C</td>
<td>Seawater / net pens with VHS-positive trout</td>
<td>3(1) x 0/3 Nd 1/1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PP / January 2009 / 2°C</td>
<td>Seawater / loading dock of slaughterhouse</td>
<td>3(1) x 0/3 Nd 1/1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PP / January 2009 / 2°C</td>
<td>Liquid waste / stunning basin</td>
<td>3 2/3 2/2 3/3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PP / January 2009 / 2°C</td>
<td>Liquid waste / bleeding basin</td>
<td>3 1/3 1/1 3/3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PP / January 2009 / 2°C</td>
<td>Liquid waste / kidney remover</td>
<td>2 2/2 2/2 2/2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PP / January 2009 / 2°C</td>
<td>Liquid waste / drain before disinfecting</td>
<td>3 3/3 3/3 3/3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PP / January 2009 / 2°C</td>
<td>Liquid waste / drain after disinfecting</td>
<td>3 0/3 Nd 0/3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PP / March 2009 / 0°C</strong></td>
<td>Seawater / loading dock of slaughterhouse</td>
<td>2 x 0/2 Nd 2/2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PP / March 2009 / 0°C</strong></td>
<td>Liquid waste / stunning basin</td>
<td>2(1) 1/2 1/1 1/1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PP / March 2009 / 0°C</strong></td>
<td>Liquid waste / bleeding basin</td>
<td>2(1) 2/2 2/2 0/1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PP / March 2009 / 0°C</strong></td>
<td>Liquid waste / kidney remover</td>
<td>2(1) 2/2 2/2 1/1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PP / March 2009 / 0°C</strong></td>
<td>Liquid waste / drain before disinfecting</td>
<td>2(1) 0/2 Nd 0/1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PP / March 2009 / 0°C</strong></td>
<td>Liquid waste / drain after disinfecting</td>
<td>Nd Nd Nd Nd Nd</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
6. Discussion

This thesis consists of several observational studies conducted at fish farms and processing plants, farming or handling VHSV-infected rainbow trout or whitefish during the study period. Also, several experimental field and laboratory challenge tests were performed. The farms were situated in a restriction area regarding VHS since 2000 with strict restriction concerning transport of live fish and farming equipment out from this area. Within the restriction area, fish farming continued despite the presence of VHS, and processed fish could be transported out from the restriction area. In the restriction area, the infrastructure of the fish farming activity did not take into account the possible spread of fish diseases. Movement of fish was conducted in a way where different age classes met several times and connection to processing plants was continuous. Efforts were however made to sanitise fish farms regarding VHS, mainly the summer farming localities that were emptied of fish every autumn. Winter farming localities were usually never completely emptied of fish and sanitised. This was typical especially for farm A, which was situated next to a processing plant and fish were continuously moved between winter locality and the nearby summer locality. The movement of fish at farm B was mostly in one direction, from fry farming to processing plants, but daily contacts from the processing plants to most of the farming localities existed due to the daily servicing of the farms. When this study was planned, it was quite clear that VHS would be almost endemic at the fish farms in the study area, which gave an excellent opportunity to study different ways of screening for the disease and factors that could affect disease eradication. In the following sections all the objectives of the study are discussed.

6.1 Surveillance of VHSV infection in rainbow trout populations (I)

In 2006, the European Commission released a new directive for its member states concerning health requirements for aquaculture and prevention and control of certain diseases including VHS (EC 2006). The disease surveillance was to be risk-based in all aquaculture areas, a demand that was not easy to implement in countries where the fish farming industry is small-scale, and no fish health services are available. Risk-based approaches specially directed to these aquatic animal diseases were not presented in this directive or other resolutions of EC at that time (Oidtmann et al. 2013). One important aspect of the risk-based surveillance is the demand that samples should always be sent in for diagnostics if any suspicion of fish disease is raised. In Finland, laboratory services, for diagnosing fish diseases have been available for decades, but there have been no fish health veterinarians working in the field to help farmers with practical health problems. Especially in food fish farming it was not a common custom to contact a veterinarian due to health issues, and samples were usually sent only during summer when bacterial diseases cause high mortalities. The reason for the lack of fish health services is that the Finnish fish farming industry is small and scattered over a large area, making private fish health services
unprofitable. In the study area, sampling for viral fish diseases was mainly conducted during official inspections, and the official screening programme was not risk-based but solely active surveillance. Active surveillance is based on the competent authority’s own activity to secure sampling and reporting according to legislation.

6.1.1 Surveillance procedures

In the first study (I), one of the surveillance programmes (programme 1) was conducted by the farmers (farms A and B), who were promised free diagnostic services if they agreed to send in samples every time they noticed signs that could indicate fish disease in their fish populations. Previously, farmers only sent samples when high mortalities were seen; mild signs of disease were often not reported or confirmed. VHSV 1d was reported to cause 40% mortalities in an infection trial (Raja-Halli et al. 2006), but such high mortalities were rarely reported in the study area (author’s unpublished data). Mortalities ranging from 10% up to 50% have only been detected after stressful events, such as the transportation of infected populations between farming localities or to processing plants.

This syndromic surveillance was the most reliable means of screening for the presence of VHSV, being up to 17 times more effective than the active surveillance in programme 4 carried out by the competent authority. This result indicates that active surveillance only using methods to detect viruses, although regarded as sufficient to show infection in an area, is not a reliable tool to reveal whether a single population is or has been infected by VHSV. It is notable that Programme 3, with sampling after a clinical VHS outbreak, performed almost as well as Programme 1, while the other two sampling programmes (2 and 4) performed less efficiently. In practice, an active surveillance procedure (programme 3) that is performed after a confirmed clinical VHS outbreak would not be a sensible strategy to identify VHSV-infected farms, as they have already been found. In contrast, syndromic surveillance outperformed active surveillance programmes and has clear practical value.

The farmers that carried out the syndromic surveillance in programme 1 had six years’ experience of VHS outbreaks and good skills in detecting abnormalities indicating a disease outbreak in the early stages of infection in their fish populations. This experience is considered an important factor in the good result of programme 1. The finding that temperature affected the performance of the programmes might be associated with this: fish farmers found the occurrence of clinical cases at certain temperatures to be typical of the disease. The staff of the study fish farms were also motivated to participate in this surveillance, and new means of transportation of samples to the laboratory were found, which was also vitally important for this field study. In programmes 2 and 3, the populations were also carefully observed at the time of sampling, but only the farmers were able to follow up their fish populations daily. Sampling implemented in the early stages of the infection when clinical symptoms can be observed have a higher possibility of finding test positives, as there are high quantities of the virus present. Sampling performed as part of active surveillance is mainly successful for testing carriers of disease with low or undetectable amounts of the disease agent.
The water temperature in the study area varies from slightly above 0°C in winter to often above 20°C in summer. In autumn, the water temperature drops below 15°C in late September, and ice may cover the farming localities from December to late April. This ice layer can make inspection and sampling impossible for several months. When the ice layer melts in spring, the water temperature often rises above 15°C within 2–3 months, varying from year to year. This gives a short time window for the authorities to visit the farms, which are scattered around thousands of small, difficult to reach islands. The fish populations are therefore often sampled at the processing plant, where disease signs are difficult to notice.

Sandlund et al. (2014) reported that gills are useful target organs in screening chronic or sub-acute VHSV infections. Therefore, it could be argued that programme 1 would not differ as much from the other programmes if the gills had also been tested in the other programmes. However, gills were used on two occasions in parallel with the other organ samples of the same fish in this study without gaining any new information (Vennerström, unpublished data).

6.1.2 Diagnostic methods used for screening

Both virus isolation in cell culture and real-time RT-PCR are reliable tests for detecting VHSV when there is an acute VHSV infection at the time of sampling, but effective early warning systems are required to detect signs of the disease. Real-time RT-PCR is a rapid and reliable test to confirm or rule out the presence of VHSV in organ suspension when clinical signs have given reason to suspect infection. Real-time RT-PCR is also reported to be valuable in finding asymptomatic fish carrying VHSV (Hope et al. 2010). In our study, real-time RT-PCR detected a possible carrier on one occasion when virus isolation in cell culture failed. This was a situation in spring 2007 when the water temperature was close to 15°C and rising. It is possible that this population had just been infected and clinical signs of VHS had not appeared before the water temperature rose above 15°C. We have noticed that outbreaks due to VHSV Id do not occur and the virus cannot be isolated at temperatures higher than 15°C. Serum samples that were collected on the same occasion did not reveal any antibodies against VHSV, also suggesting an early infection. Our suspicion concerning the carrier state was confirmed the next autumn, when the water temperature dropped below 15°C and the fish in this population experienced a clinical disease outbreak. Serum samples taken at this time revealed only one positive sample out of 15 tested. This suggested a new infection and indicated that the virus infection did not have time to spread in the population before the water temperature rose above 15°C during the previous spring.

Real-time RT-PCR is valuable when screening for a particular virus, e.g. in wild fish, and where a positive signal does not lead to legal actions against the business owner. Real-time RT-PCR could also be used as the primary diagnostic screening test for fish farms, but a positive result should be confirmed using other methods such as sequencing of positive products and molecular epidemiology. Use of serological testing of antibodies against VHSV from fish serum is also possible.

Screening for antibodies against VHSV using ELISA and PNT has been reported as useful methods in surveillance of VHSV-infected populations (Fregeneda-Grandes and
Olesen 2007, Fregeneda-Grandes et al. 2009, Schyth et al. 2012, Millard et al. 2014, Wilson et al. 2014). In our study, we tested fish sampled in programmes 2 and 3 for antibodies against VHSV using an ELISA method. We detected antibodies against VHSV several months after a clinical disease outbreak had occurred. However, the results were easy to interpret only if there had been a clear clinical outbreak no more than one year earlier in the population.

Early detection of VHS is essential for successful disease eradication. Virus infections are easily spread between farms in the same area due to daily management practices. Routine clinical inspections performed by skilled fish health specialists have also been noted as essential in the surveillance of freedom from VHS in Norwegian marine salmon farms (Lyngstad et al. 2016). Our study supports the Norwegian report by demonstrating that more frequent monitoring for clinical signs of VHS outperforms active surveillance. There are no fish health services offering routine clinical inspection or sampling services for the fish farms in the study area. The farming localities are difficult to reach and shipping of samples by the farmers themselves for testing of diseases is complicated, as the logistics involved in transferring samples between the study area and the laboratory are often poor.

The successful eradication of VHSV in two other areas on the south coast of Finland in 2001 and 2003 (reinfection 2008) could be explained by the early detection and rapid eradication of the affected farming localities, which is vital for the eradication of and subsequent freedom from disease. Farmers contacted the authorities immediately when they noticed suspicious disease signs. Eradication was performed without delay and in good cooperation between farmers and authorities. In Åland, this cooperation was not as successful at the beginning, VHSV rapidly spread between farming localities and stamping out the disease was not economically justified. We believe that this study managed to improve the screening of VHSV and biosecurity measures in this area. According to the official disease surveillance in the restriction area in Åland, the number of VHS-positive samplings has followed a decreasing trend (ICES 2014), which indicates a lower infection pressure in the area. VHSV has not been isolated in the study area or anywhere in the Åland Islands since 2012.

6.2 Studies on wild fish and farmed whitefish (II)

6.2.1 Wild fish

Wild fish have been reported to be carriers of several different VHSV genotypes in the North Sea and the Baltic Sea (Skall et al. 2005ab). However, there are no reports indicating that wild fish would relate to the clinical disease caused by VHSV genotype Id in farmed fish. The number of different fish species in the close vicinity of the fish farms in this study is high, but the number of individuals of each fish species varies from only a few individuals like pike or sea trout to thousands like perch, roach or three-spined stickleback. In our study, the observed prevalence of VHSV was zero in 17 different tested wild fish species that were
screened during several years in the vicinity of two infected fish farms. Additionally, wild perch and roach did not become infected when they were challenged with VHSV by keeping them in small cages close to rainbow trout experiencing a clinical outbreak of VHSV.

Wild fish were not found to be a likely source for the reappearing VHS outbreaks in the Finnish brackish water fish farms. The results indicated that if the screening had missed the infection in species caught in quite high numbers the prevalence would have been no more than 4%. The sample size was low for defining the possible prevalence range of some species and the results are inconclusive for them. These fish species that were caught in small numbers comprise less than 5% of all fish analysed. The species in question have a low apparent prevalence on the farms and in their vicinity. In addition, a fish farm is not a normal habitat for these fish species and even a small number of these species probably represent a sufficient portion of the individuals present. Organ pools from fish species caught in low numbers contained less individuals than species caught in high numbers, reducing the possible effect of virus dilution. It is possible that some of the tested fish species could be transient carriers of the virus and therefore not caught by the screening method used. More studies on the prevalence of VHS in wild fish are needed, as all fish catchment methods have species and size selectivity that may induce bias in the results.

The minor role of the wild fish being the source for reappearing VHS outbreaks in farmed rainbow trout in the study area is supported by several facts. The farms where the wild fish were caught experienced several outbreaks of VHS in their fish during the study. If the wild fish had a major role, one would expect to find clear positive signals with real-time RT-PCR from the tested wild fish, as VHSV was present in the environment on several of the sampling occasions. Another fact indicating the minor role of wild fish is that VHSV was successfully eradicated at the first attempt from the other two restriction areas regarding VHSV in Finland with similar farms producing rainbow trout and the same kind of wild fish populations as the farms in the study area in the Province of Åland.

Further support for the minor role of wild fish is that extensive screening of wild herring and sprat has been conducted on the coast of Finland without finding any samples positive for VHSV Id (Gadd et al. 2010). It was believed that herring on their spawning migration would have brought the infection to Finnish fish farms in the same manner as was reported on the west coast of Sweden in 2000 (Nordblom and Norell 2000). In Sweden, the same type of VHSV Ib was isolated from both herring caught close to infected rainbow trout farms and from the rainbow trout on these farms. It was assumed that the herring on their spawning migration brought the infection repeatedly to the farm and eventually made rainbow trout farming in that area impossible (Nordblom and Norell 2000, Jansson and Vennerström 2014). This has not been the case in Finland, and there is no indication that eradication measures should be omitted because of wild fish.

**6.2.2 Farmed whitefish**

In our study, farmed whitefish, challenged in the same way as perch and roach, were infected by VHSV Id and the virus replicated in the tested organs. The virus could be detected for a short period, but no mortalities were recorded. Our results are supported by
the report from Skall et al. (2004a) of an infection trial with farmed whitefish where they found whitefish to suffer only low mortality after infection with a VHSV Ib strain isolated from marine fish. They also found that infected fish continued to carry the virus for at least the three weeks that the trial lasted. VHSV has also been reported in whitefish in Switzerland and Germany (Ahne and Thomsen 1985, Meier et al. 1986).

Whitefish had become a quite common farmed food fish species in the study area a few years before VHSV was detected for the first time in Åland in 2000. Whitefish was often farmed on same farms as rainbow trout, but according to the Finnish authorities, VHSV has only once been isolated from farmed whitefish in the restriction area regarding VHSV (Vennerström, unpublished data). Whitefish should be considered potential carriers of VHSV and a source of the recurring VHS outbreaks in the VHS-restriction area of Åland. Whitefish is a native species of the Baltic Sea, where VHSV is endemic. According to phylogenetic studies undertaken by Einer-Jensen at al. (2004) the Finnish rainbow trout isolates (VHSV Id) are closest to the common ancestor of marine (VHSV Ib) and freshwater isolates. Perhaps whitefish that is a native fish species of the Baltic Sea has developed a genetic resistance to marine strains of VHSV and is therefore not as sensitive to the disease as the imported non-native rainbow trout. This is supported by the fact that mortality caused by VHSV Id in Finnish whitefish has never been reported, although whitefish are also screened for VHSV repeatedly according to regulations set up by the competent authority.

Since whitefish may be infected by VHSV Id and the virus can replicate in this fish species, whitefish kept close to VHSV-positive rainbow trout populations may give the virus an opportunity to ‘jump’ to whitefish and survive longer in the area. This may also give the virus an opportunity to adapt and become more virulent to whitefish if given the opportunity to jump between species by farming several fish species on the same farms or close to each other. This may have been the case in Norway, where VHSV of genotype III was isolated from farmed rainbow trout on the west coast in 2007. Genotype III could be considered endemic among wild fish e.g. cod in the North Sea (Snow et al. 2000, King et al. 2001, Smail 2000). According to earlier infection trials, rainbow trout has not been sensitive to this genotype (Skall et al. 2004b). In 2007, a new type of VHSV genotype III was isolated from rainbow trout that was pathogenic to rainbow trout (Dale et al. 2009). The source of the infection is not clear, but these positive farms had close connections to cod (Gadus morhua) and saithe (Pollachius virens) farming where raw processed fish of marine origin were used for feed. Containers of dead farmed rainbow trout, cod and saithe from other localities were stored close to the primary infected locality (Dale et al. 2009).

It could also be possible that whitefish can be transient carriers of VHSV, as the virus was not detected in the whitefish groups after the infected rainbow trout were mowed away from the farm.

6.2.3 Sprivivirus

Sprivivirus was isolated from all tested fish species (perch, roach and whitefish) in the infection trial, but was not associated with mortalities. These findings were not studied further in this trial. Sprivivirus has occasionally been isolated from farmed sea trout in the
study area in connection with bacterial fish diseases during the summer when water temperatures are over 15°C (personal communication Holopainen R.). Sprivivirus seems to be an endemic virus in the study area and it is not clear whether this virus could be a predisposing factor for the VHSV outbreaks or influence the screening of VHSV. Sprivivirus has not been reported from the two successfully eradicated restriction areas of VHS in Finland.

6.3 VHSV in the environment of fish farms and processing plants (III)

6.3.1 Blue mussels

Based on the results from our studies on blue mussels, it can be assumed that the VHSV is not able to replicate in blue mussels and is quickly inactivated in them. This was shown by taking samples from the hepatopancreas of mussels living in VHSV-infected fish farms and by two different infection trials using high doses of VHSV. The challenges were performed using two different methods, but the result was the same regardless of the method used. The rapid inactivation of VHSV in sea water, but somewhat longer persistence of VHSV RNA in mussels, was observed in our study (especially in the second challenge test). Blue mussels may serve as a physical attachment surface for VHSV. Thus, it is possible that mussels can protect VHSV in sea water from environmental effects that could destroy the virus and may prolong the viral contamination of the environment even if the fish farms are fallowed. The difference could be a result of the frequent water changes in the test aquariums that were performed to give the mussels as good conditions in the aquarium as possible. VHSV is an enveloped virus that is not as resistant to environmental effects as birnaviruses, which have no envelope and have been found in free-living molluscs (Mortensen et al. 1992, Rivas et al. 1993, Bovo et al. 2005). However, the replication of VHSV in mussels was unlikely, otherwise increased secretion of the virus would have occurred in the mussels and one would have expected the virus load in the aquarium water to increase as well. The result is also indirectly supported by the fact that VHS was successfully eradicated in two similar farming localities farming rainbow trout for consumption on the west and south coast of Finland (Raja-Halli et al. 2006). These farms also had high densities of blue mussels in their environment. If VHSV could replicate in mussel tissues, one would expect those eradications to have failed. Similar results have been shown in studies with infectious salmon anaemia virus ISAV (Skår and Mårtensen 2007).

6.3.2 Liquid waste and sediment

VHS virus was more frequently detected in sea water close to the net pens with VHSV-diseased rainbow trout populations and in liquid waste from processing plants handling
VHSV-positive fish in cold water temperatures during winter than in spring. The water temperature in the study area was close to 0°C in January–March and 4–10°C in April–May. Daylight is only 6 h in January but increases to 14–16 h in April–May (Nordlund 2017, Cornwall et al. 2018). The low amount of UV radiation in winter (Finnish meteorological institute 2019) in the study area together with the short daylight hours and cold-water temperature could explain the difference in virus survival. The result is consistent with previous studies where VHSV was reported to be sensitive to UV light and to survive longer in cold water temperatures than in warm (Ahne 1982, Parry and Dixon 1997, Øye and Rimstad 2001, Yoshimizu et al. 2005, Hawley and Garver 2008).

Liquid waste samples from the processing plant collected in March 2009 were positive for VHSV RNA, although only clinically healthy whitefish had been processed at the time. Although these whitefish were not sampled in this study, we have noticed in a previous study that although whitefish are not easily infected with VHSV genotype Id, some fish in the population may become infected and virus replication occur (Vennerström et al. 2018). The processed whitefish were farmed next to the processing plant where VHSV-positive rainbow trout had been processed earlier the same year, and it is possible that the virus was transmitted. Another possibility for this virus-positive finding is that the processing line was highly contaminated by VHSV RNA from infected rainbow trout processed earlier. It could well be assumed that processing plants handling VHSV-positive fish and the surrounding environment are heavily contaminated with the virus, especially in winter. For this reason, any contact between these plants and susceptible farmed fish populations should be avoided, especially during the coldest and darkest time of the year.
7. Conclusion

Syndromic surveillance (based on the observation of clinical disease signs in fish by fish farmers) is more sensitive than active surveillance when detecting VHSV infections on fish farms. Active surveillance (programmes 2, 3 and 4) did not yield information that would have been needed for preparing eradication plans in the VHS-eradication area of Åland.

Real-time RT-PCR was at least as reliable as virus isolation in cell culture to detect infection in this study. Serology proved to be a useful test to determine whether a fish population had been infected with VHSV. However, the antibody levels are very low in mild disease outbreaks or if the infection has occurred several months earlier. Therefore, the use of this antibody test in screening for VHSV in disease-free areas is not reliable without affirmation of the test results using another test, such as virus isolation or PCR. On the other hand, it is a useful additional tool in VHSV eradication for screening populations during the follow-up period, before declaring an area free of infection.

Processing plants handling VHSV-positive fish and sea water close to VHSV-positive fish populations are contaminated with VHSV especially during winter when daylight is sparse, and water temperatures are close to zero. Contact with contaminated localities increases the risk of the disease spreading to susceptible fish populations. Based on our results, blue mussels are not a relevant source of VHSV, as the virus is rapidly inactivated in mussel tissues, but they could provide VHSV with a physical protective environment that could prolong the survival time of the virus, although probably not for more than a few days.

According to our study, wild fish living freely in the fish farming area do not seem to threaten the farmed fish with respect to VHSV genotype Id infection in Finland. Farmed whitefish as a native species was a possible source of the recurring VHS outbreaks in Finnish brackish water food fish farms, as they were infected but seemed to clear out the infection. Wild fish may function as carriers of virus between closely situated farms. Therefore, it is important to perform quick stamping-out of infected fish farms and decrease the infection pressure and adaptation possibilities in other fish species.

Early detection of VHSV infection is crucial for VHS management. The personnel working on fish farms have the key role in disease management as they are the only persons that can notice even small changes in their fish populations, indicating a possible infectious disease. In addition, it is important to have a good cooperation between farmers and the fish health specialist so that even small suspicions would lead to testing for possible infections.
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