Genetic variation of *Piscine orthoreovirus* and the presence of HSMI in farmed Atlantic salmon from Arctic Norway

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Title: Genetic variation of *Piscine orthoreovirus* and the presence of HSMI in farmed Atlantic Salmon in Arctic Norway

Abstract

Piscine orthoreovirus-1 (PRV1) is ubiquitous throughout the world and can cause heart- and skeletal muscle inflammation (HSMI) in farmed Atlantic salmon (*Salmo salar*). HSMI was first diagnosed in Norway in 1999. The virus is highly prevalent among farmed salmon in Norway, but the pathogen doesn't always induce HSMI. PRV1 groups in two major clades, PRV1a and PRV1b, which is believed to vary in virulence and severity of disease. The putative low virulent PRV1a is dominating in North American Pacific Coast (NAPC) where only mild, or no lesions have been described from infected salmon. By looking at previously sequenced isolates from Norway, the putative high virulent PRV1b is dominating with one PRV1a isolate from 1988. Severe inflammation in heart- and skeletal muscle known as HSMI is frequently diagnosed in Norway. Virulence is previously linked to the viral proteins encoded by S1 and M2 segment, respectively. The aim of this study was to identify the genotypes of PRV1 associated with heart- and skeletal inflammation (HSMI) in farmed Atlantic Salmon in Northern Norway.

Phylogenetic and sequence analyses, of segment S1 and M2, were performed on 58 PRV1 sequence isolates collected from the production sites in this study with the aim to further elucidate the linkage between the segments and the virulence of the genogroup.

The results in the study confirms and further strengthen the classifications which differentiates PRV1 in two subgenotypes, PRV1a and PRV1b. Both clades were shown to be present in farmed salmon in the arctic region of Norway. PRV1b was shown to be the dominating clade. In sites where PRV1a was present its prevalence varies and was found to be 10.5 % at most. The actual importance of this in the field is unknown and needs to be explored further. The genetic variations of PRV1b were less significant with a few exceptions. Both clades are present in farmed Atlantic salmon in Arctic Norway. The prevalence of HSMI is overall high, regardless of which genotype is present at site. One site with PRV1b did not experience elevated mortality because of HSMI, which may indicate that other factors, as environmental factors are essential for development of HSMI.

Abbreviations

CMS	Cardiomyopathy syndrome
EIBS	Erythrocytic Inclusion Body Syndrome
HSMI	Heart- and skeletal muscle inflammation
NGS	Next generation sequencing
ISVP	Infectious subviral particles
SAV	*Salmonid alphavirus*
PD	Pancreas disease
PMCV	*Piscine myocarditis virus*
PRV	*Piscine orthoreovirus*
PRV1a	Putative low virulent variant of PRV1
PRV1b	Putative high virulent variant of PRV1
MRV	*Mammalian orthoreovirus*
ISAV	*Infectious salmon anemia virus*
ISA	Infectious salmon anemia
IPNV	*Infectious pancreatic necrosis virus*
IPN	Infectious pancreatic necrosis
NAPC	North American Pacific Ocean
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction (qPCR)
Ct	Cycle threshold
NTC	Non-template control
PCR	Polymerase Chain Reaction
Virus “isolates”	a term used for sequence isolates, to separate it from virus isolates where all segments are isolated.

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1. Introduction

1.1 Norwegian aquaculture

Norway is the largest producer of Atlantic salmon (*Salmo salar* L.) in the world, with a total of 1 364 044 tons produced in 2019 (Statistics Norway (SSB), 2020). The Norwegian salmon farms are located along the entire western coastline, from Rogaland in the south, to Finnmark in the north. Salmon are farmed in an industrial scale with high densities making them vulnerable to several risk factors that impact mortality, growth rate, and harvest quality. Disease outbreaks are a special concern as they reduce salmon welfare and have a negative economic impact on production through loss of biomass and harvest quality (Ebert & Bull, 2003). Since the start of salmon farming in the 70's, both bacterial and viral diseases have caused challenges for the industry. In the 90's, oil adjuvant injection vaccine was introduced allowing for mitigation of most common bacterial diseases without the use of antibiotics (Sommerset, Krossøy, Biering, & Frost, 2005). Diseases of viral etiology have continued to cause disease challenges as they lack the prophylactic measures and treatments available for bacterial diseases (Aldrin, Rd Storvik, Frigessi, Viljugrein, & Jansen, 2009).

1.2 Viruses associated with muscle pathology

Viruses have been identified to be the causative agent of several of the most important salmon diseases associated with pathology affecting the filet quality and general downgrading in the salmon market (Färber, 2017). Melanized focal spots, referred to as "black spots", occurring in the salmon filet is considered one of the most severe quality challenges for the salmon industry in Northern Norway (Nordberg, 2018). It is likely that melanized spots are affected by several factors including handling, vaccination, feed and diseases (Bjørgen et al., 2019, 2015; Koppang, Haugarvoll, Hordvik, Aune, & Poppe, 2005; Mørkøre, Ytrestøyl, Ruyter, Torstensen, & Thomassen, 2014). Other challenges concerning the filet quality are viruses affecting the muscle pathology. The three viral pathogens *Piscine orthoreovirus* (PRV), causing heart- and skeletal muscle inflammation (HSMI), *Salmonid alphavirus* (SAV) causing pancreas disease (PD), and *Piscine myocarditis virus* (PMCV) causing cardiomyopathy syndrome all results in heart pathology in the infected salmon, the two former are also causing muscle pathology (Finstad, Falk, Løvoll, Evensen, & Rimstad, 2012; Garseth, Fritsvold, Svendsen, Bang Jensen, & Mikalsen, 2018; Moriette, LeBerre, Boscher, Castric, & Brémont, 2005). PD is not present

in the northern most parts of Norway. This is in contrast with both PRV, PMCV and melanized focal changes that are found in farmed salmon in all production areas in Norway.

1.3 PRV

PRV1 have been identified in farmed salmonids in Norway, Scotland, Canada, Sweden, Denmark and Chile (Di Cicco et al., 2018; Ferguson, Kongtorp, Taksdal, Graham, & Falk, 2005; Godoy et al., 2016) and seems to be highly host specific (Polinski, Vendramin, Cuenca, & Garver, 2020). The virus is typically detected 5-9 months after sea transfer (R. T. Kongtorp, Kjerstad, Taksdal, Guttvik, & Falk, 2004a). It is also detected in freshwater sites, where it can be challenging to get rid of (Hjeltnes, Bang-Jensen, Walde, Haukaas, & Walde, 2019). The most likely introduction of PRV1 into freshwater sites are through biological material. It will most likely infect the eggs by contaminations from the blood of infected brood fish and follow the fish throughout the production cycle (Løvoll et al., 2012; Polinski et al., 2020). PRV1 cause HSMI in challenge trials, but there is no elevated mortality, in contrast to observations done in the field (Wessel et al., 2017). PRV1 can most likely also cause jaundice/anemia in Chinook salmon (*Oncorhynchus tshawytscha*) in British Colombia (Di Cicco et al., 2018; Miller, Günther, Li, Kaukinen, & Ming, 2017).

PRV2 has only been detected in Japan, and is associated with the blood disorder known as erythrocytic inclusion body syndrome (EIBS) in farmed Coho salmon (*Oncorhynchus kisutch*) (Takano et al., 2016). The syndrome has been reported to occur in both freshwater and seawater production (Okamoto, Maita, Ikeda, Takahashi, & Rohovec, 1992). The disease can cause a significant reduction in the hematocrit levels in the fish. In experimental challenges the virus have led to moderate anemia (Takano et al., 2016). Clinically EIBS can cause jaundice in diseased Coho salmon, where bilirubin accumulated in the liver causing a yellow discoloration in the fish (Sakai et al., 1994).

In Norway PRV3 was first discovered in farmed Rainbow trout (*Oncorhynchus* mykiss) with clinical disease similar to HSMI showing circulatory failures and similar histopathology, but the natural host seem to be trout, *Salmo trutta* (Garseth et al., 2019; Kuehn et al., 2018; Olsen, Hjortaas, Tengs, Hellberg, & Johansen, 2015; Sørensen et al., 2020). This virus seems to be host specific and rarely detected in Atlantic salmon (Olsen et al., 2015). The virus has been

detected in at least six European countries as well as in Chile. The outcome of an infection with PRV3 seems to be affected by the farming environment, as the disease has been associated with aquaculture in both Norway and Denmark. In experimental infection trails the virus failed to induce a persistent infection (Vendramin et al., 2019). The Norwegian variant can cause HSMI-like disease (Olsen et al., 2015).

1.4 Diseases caused by PRV

Various diseases have been associated with PRV. EIBS, HSMI and HSMI-like disease have been connected to high mortality, muscle pathology and unfavorable fish welfare worldwide. EIBS can cause severe anemia and mass mortality in farmed Coho salmon in Japan, and in turn this can cause great economical losses (Okamoto et al., 1992). This disease has also been reported from Chinook salmon. It is demonstrated to have variable susceptibility among salmonids (Okamoto et al., 1992). The causative agent of EIBS have been shown to be PRV2 (Takano et al., 2016).

HSMI mortality range from none to as high as 20% in affected pens. This is often combined with a morbidity as high as 100% (R. Kongtorp, Taksdal, & Lyngøy, 2004b). HSMI can cause both loss of biomass and reduced product quality and therefore have the potential to cause severe economic impact both on site level and in the salmon industry. The disease is present in all of Norway`s production areas and is considered as severe because of the lack of an available treatment (Sommerset, Jensen, Bornø, Haukaas, & Brun, 2021). HSMI is mainly characterized by signs of circulatory failure, and histopathological findings including panmyocarditis, lesions in the red skeletal muscle and multifocal necrosis of hepatocytes in the liver (R. Kongtorp et al., 2004b). PRV1 have been shown to cause HSMI (R. T. Kongtorp et al., 2004a; Wessel et al., 2017). This was shown by experimental challenges using viral particles originating from blood of PRV1 infected salmon and not through cell culture (Wessel et al., 2017).

HSMI-like disease This disease has only been observed in the *Oncorhynchus* species. In 2013 several disease outbreaks occurred in Rainbow trout at freshwater hatcheries in Norway. The causative agent was unknown, later found to be PRV3 (Dhamotharan et al., 2018; Hauge et al., 2017). The fish showed sign of circulatory failure, and histopathological findings as pancarditis in the heart, lesions in the red skeletal muscle and focal, partly confluating vacuolization in the

liver (Olsen et al., 2015). The mortality was moderate to high, and the disease was also observed in the fish after sea transfer (Hauge et al., 2017).

All three of the diseases have been associated with PRV. PRV is widespread throughout the world and can cause great consequences for salmon farming. It can be divided into three genotypes which all gives different, but somewhat similar diseases affecting the circulatory system.

1.5 HSMI in Norway

The HSMI-situation in Norway is considered severe with a high impact on fish health. The first reported case of HSMI in Norway was from a salmon farm in Trøndelag county in 1999 (R. T. Kongtorp et al., 2004a). Since then, the disease is considered ubiquitously throughout the country. It was classified as a notifiable disease, at the Food and Safety authority, from 2004 up until 2014. The highest number of cases occurred in 2014 with 181 diagnoses of HSMI (Bornø & Lie, 2015). The high number of cases, a high prevalence of PRV1 virus in salmon production and a lack of prophylactic measures, resulted in the removal of the disease from the list of reportable salmonid diseases in Norway (Bornø & Lie, 2015). Today HSMI/PRV is not notifiable to any international, national, or regional governing bodies (Polinski et al., 2020).

The increased use of private diagnostic laboratories by Norwegian aquaculture and the removal of HSMI from the list of notifiable diseases resulted in the loss of a complete overview of official diagnoses of HSMI. It is believed that this have led to a steady drop in annual cases, and it is suspected that HSMI is highly underreported because of the lack of input from the private laboratories. The annual cases of HSMI have continued to decline, and in 2019 it was the lowest in 10 years with 79 cases (Sommerset et al., 2020). In 2020, it was reported 161 cases, a significant increase from the prior year, most likely caused by improved sharing of data between The Veterinarian Institute (VI) and some of the private laboratories. This led to a more holistic collection of the reported detections of the disease. Still, the collaboration only provides 75% of the diagnostics in the country, so the number is probably even higher (Sommerset et al., 2021). The surveillance of HSMI is minimal in most countries, as the disease is most common in Norway (Wessel et al., 2018). In Norway, the pathogen is frequently tested by RT-qPCR for as a part of routine diagnostic work when investigating a disease outbreak.

The focus on certifications is growing, and Aquaculture Stewardship Council (ASC) published a standard for salmon aquaculture in 2012 to ensure fish welfare. One criterion is that the certified farm must keep a detailed record of all mortalities and classify a cause of death of all fish post-mortem. One such classification is “circulatory failure”, and this classification is for seemingly flawless fish. This is a simplification which is manageable for the workers at the facility to classify. Fish with HSMI will be placed in this classification (ASC, 2012).

1.5.1 Clinical signs and gross pathology

HSMI is characterized by inflammation in the heart and skeletal muscle, where outbreaks can results in high mortality up to 20 % in the affected pen and 100 % morbidity in the affected facility (R. Kongtorp, Taksdal, & Lyngøy, 2004b). Fish with HSMI are often positioned near the walls of the net pen and oriented towards the current. The fish can become lethargic and be perceived as slow with an abnormal swimming pattern, and with few other clinical signs (R. T. Kongtorp, Halse, Taksdal, & Falk, 2006; Watanabe et al., 2006). The internal clinical signs are typical of a circulatory failure. The heart is pale with a loose texture. Ascitic fluids in the pericardial and perivisceral cavities is often present, as is pericardial hemorrhage. The liver can be covered by a white fibrinous layer in severe cases. The spleen is swollen, and petechia in the perivisceral fat may occur. A decrease in appetite results in an empty gut (R. T. Kongtorp et al., 2006, 2004a&b).

1.5.2 Histopathology

Classical histological findings are found in the heart and red skeletal muscle. Infection gives lesions in the ventricular compactum, epicarditis, focal myocarditis and endocarditis. This can lead to an extensive pancarditis with infiltration of inflammatory cells, as macrophages and neutrophils, in the epicardium and both spongy and compact myocardium (Bruno, Noguera, & Poppe, 2013). In the red skeletal muscle, there can be vacuolation, loss of striation, infiltration of mononuclear inflammatory cells and necrosis. Lesions in other organs are few, but the liver can suffer from multifocal necrosis with vacuolated and pyknotic or karyolitic cells (Bjørgen et al., 2015; Bruno et al., 2013; R. Kongtorp et al., 2004b).

1.5.3 Diagnostic methods and treatments

When observing fish that has characteristic signs of disease, samples must be taken and analyzed for HSMI. The gross pathology, histological changes and detection of the pathogen are all important in setting a HSMI-diagnosis.

Tissues as gills, heart or kidney can be analyzed for the presence of PRV1 by real time RT-PCR (qPCR). There are several important differential diagnoses that have a similar clinical presentation, such as CMS, ISAV and PD. Both ISAV and PD are notifiable list 3 diseases. HSMI can occur in a coinfection with each of these diseases (R. Kongtorp et al., 2004b). To differentiate HSMI from the other diseases the combined use of histopathology and qPCR is necessary. The type of cardiac lesions, pancreatic lesions and pathological changes in red muscle are all characteristic for the individual diseases (Bruno et al., 2013).

As there is no efficient vaccine available for HSMI, other prophylactic measures have been used to reduce the impact of the disease and spread of PRV1; such as reducing stress and avoiding mixing of populations. One measure that has been suggested to mitigate PRV from freshwater sites is through screening eggs and broodfish and discarding positives, currently this is not standard practice (Wessel, Hansen, Løvoll, et al., 2020). The use of feed with higher EPA levels have been shown reduce the severity of the HSMI pathology (Hatlen et al., 2016). Also the use functional feeds have been suggested to have effect but feeds does not reduce prevalence or clear viral infection of PRV1 (Grammes, Rørvik, & Takle, 2012; Martinez-Rubio et al., 2012). The normal progression of HSMI is that mortality will normalize after the disease outbreak and the salmon population recover over time (Løvoll et al., 2010)

1.6 Piscine orthoreovirus

1.6.1 Taxonomy

Piscine orthoreovirus is a species in the genus *Orthoreovirus,* family *Reoviridae* (Markussen et al., 2013; Palacios et al., 2010). Reoviruses have a dsRNA genome, which was first described in 1963 (Gomatos & Tamm, 1963). Reoviruses are non-enveloped with a icosahedral symmetry and have been reported to infect a wide range of species such as mammals, birds, reptiles etc. (Attoui et al., 2012). *Reoviridae* is divided into two subfamilies: *Sedoreovirinae* and *Spinoreovirinae*. Some of the characteristics of *Spinoreovirinae* are large spikes and turrets at the surface, and the subfamily contains nine genera, including *Orthoreovirus*. Viruses in this genus have been shown to infect birds, mammals, and reptiles. It has been hypothesized that viruses in this genus have the ability to co-speciate based on the sequence identity and mutation rate among *Orthoreoviruses* (Geoghegan, Duchêne, & Holmes, 2017).

PRV was the first discovered *Orthoreovirus* to infect fish. The virus was identified through next-generation sequencing (NGS) in 2010 (Palacios et al., 2010). The PRV group meet the criteria for classifying as an species of the *Orthoreovirus* by ICTV, based on the identity percentage of the nucleotide and amino acid, and is recognized as one (Attoui et al., 2012; Max L. Nibert & Duncan, 2013). *Orthoreovirus* splits into fusogenic and non-fusogenic viruses. PRV is a non-fusogenic virus by its lack of FAST proteins, as its mammalian counterpart *Mammalian orthoreovirus* (MRV) (Key, Read, Nibert, & Duncan, 2013). PRV is differentiating from other *Orthoreoviruses* as they have a distinct cytotoxic non-fusogenic, integral membrane protein (p13), as well as an outer fiber protein, σ1, that is dissimilar compared to other viruses in the genus (Key et al., 2013).

1.6.2 Ultrastructure, segments, and proteins

The virion of PRV has an icosahedral, non-enveloped double layered capsid with a diameter at about 70 nm (Finstad et al., 2014; Wessel et al., 2017). The genome consists of 10 double-stranded, linear segments of RNA. The segments are grouped in three classes based on the size; Large (L1-L3), Medium (M1-M3) and Small (S1-S4), coding for proteins in the classes of λ, μ, and σ. Each of the segments encodes at least one protein but some of the segments are polycistronic (Markussen et al., 2013).

The inner core of the virus consists of λ1, λ3, μ2 and σ2 proteins (Joklik, 1981). The outer capsid consist of μ1 and σ3, and these proteins are structurally coupled in an T=13 symmetry and forms a hexamer (Liemann, Chandran, Baker, Nibert, & Harrison, 2002). The L1-segment codes for λ3, RNA dependent RNA polymerase. This enzyme takes part in the replication of the viral genome and mRNA synthesis (Joklik, 1981). L2 codes for the protein λ2, the capping enzyme with guanylyltranferase and methyltransferase necessary for 5'-capping of mRNA (Reinisch, Nibert, & Harrison, 2000). L3 codes for λ1 which forms the inner shell of the capsid. The protein has helicase, NTPase and RNA triphosphate activities (Dryden et al., 1993). M1, M2 and M3 repetitively codes for the proteins μ2, μ1 and μNS. μ1 is a protein in the outer capsid and is crucial for membrane penetration of the target cell (Markussen et al., 2013). μNS is the protein in PRV that organize the viral factories in the cytoplasm. These inclusions have a globular structure similar to those observed in connection with MRV (Haatveit et al., 2016; Parker, Broering, Kim, Higgins, & Nibert, 2002). The S1 segment is bicistronic and encodes both the outer capsid protein (σ3) and the p13 protein, which is a cytotoxic integral membrane protein (Wessel, Nyman, Markussen, Dahle, & Rimstad, 2015). S2 codes for the inner capsid structure protein σ2. S3 encodes for the σNS protein that forms viral factories with μNS proteins (Becker, Peters, & Dermody, 2003). The last segment, S4, codes the σ1, the outer fiber protein which is important for the cell attachment (Lee, Hayes, & Joklik, 1981).

1.6.3 Replication

Replication of PRV and its mechanisms are comparable to MRV, where the pathways are well characterized. σ1, the cell attachment protein of MRV binds to sialic acids and gets internalized by a clathrin-coated endosome (Chappell, Gunn, Wetzel, Baer, & Dermody, 1997; Maginnis et al., 2008; Silverstein & Dales, 1968). When the outer proteins μ1 and σ3 gets cleaved, it mediates the entry and disassembly of the virion, and this gives infectious subviral particles (ISVP). These are viral particles of the core which are transcriptionally active (M. L. Nibert, Furlong, & Fields, 1991). These particles ensure the viral genome to never be exposed to the cytoplasm of the host, and this helps the virus to avoid activating the immune response of the host. The morphogenesis occurs in inclusion bodies in the cytoplasm (Urbano & Urbano, 1994). The mRNA is synthesized in the subviral particles, and capped with 5'methylation (Furuichi, Morgan, Muthukrishnan, & Shatkin, 1975). The mRNA is translated by using the host translational machinery. The core viral particles are assembled in viral factories made by the

non-structural proteins. The factories packs the -ssRNA in to a particle and the +ssRNA is synthesized to form the dsRNA genome of the virus (Acs et al., 1971). The mature PRV particles are released from the cell with or without lysis, and the virus enters the host through a fecal-oral route (Hauge et al., 2016).

1.6.4 Evolution and reassortments

The virulence of reoviruses have been altered by mutations, reassortment and recombination, as well as horizontal gene transfers due to interactions with the host or other viruses (Liu et al., 2017). During the replication there will be produced mutants that may help improve the viral fitness and adaptions to new environments. These mutations contribute to the diversity of the virus (Hanada, Suzuki, & Gojobori, 2004). Reassortments and recombinations can occur when multisegmented viruses co-infect the same host (Lowen, 2018; Varsani, Lefeuvre, Roumagnac, & Martin, 2018). Reassortments are generated during co-infection and viral replications by packaging of new progeny with compatible segments from different viruses. Recombinations can be formed when the virus packs compatible segments from another virus, generated by a switch of template, which results in the related segment to take the place of the original segment (Varsani et al., 2018). These traits are important in the repairing of defective RNA or when a mutation in the genome is deleterious (Aguilera & Pfeiffer, 2019). This mechanism may have contributed to the genetic variations found in PRV1 isolates, which group the in two distinct clades, PRV1a and PRV1b (Dhamotharan et al., 2019). PRV1a is found in the North American Pacific Coast (NAPC) and is widespread through this region. There have been found fish with mild HSMI histopathological lesions in British Colombia (BC), but there were no elevated mortalities (Di Cicco et al., 2017). Tissue homogenate from fish with PRV1a have been used in challenge trials and injected intraperitoneal in Atlantic salmon. One study did not induce any signs of HSMI, but other studies reports that the PRV1a genotype induces mild lesions similarly to HSMI (Di Cicco et al., 2018; Polinski, Marty, Snyman, & Garver, 2019). Both genotypes are present in Norway, but the prevalence is unknown. The PRV1b-variant is in great contrast to the PRV1a-variant, as this variant is considered more virulent and resulting in a more severe disease outbreak (R. T. Kongtorp et al., 2004a). Norwegian strains (PRV1b) injected intraperitoneal in salmon in experimental setup have been shown to induce histopathological changes consistent with HSMI, without the elevated mortalities (Wessel et al., 2017). This contrasts with the classical HSMI outbreak described from sea sites with lethargic fish, elevated mortality, and clear histopathological findings in affected organs. This difference could be due

to differences in several factors, such as virus, host, environment, stressors, or a combination of these (Dhamotharan et al., 2019).

Dhamotharan et. al. (2019) explored the genetic diversity among 31 PRV1 isolates from farmed Atlantic salmon from different areas. The phylogenetic analyses of the concatenated full genome acid sequences showed a clear clustering of the isolates. The NAPC-isolates, Faroes-isolates, and one isolate from Norway (NOR-1988) clustered together in one clade, the suggested low virulent clade. The Norwegian isolates and one isolate from Chile clustered in another clade, the suggested high virulent clade (Dhamotharan et al., 2019). By comparing the isolates segment by segment some of the Norwegian isolates moved between the two clades, which could be explained by reassorting in the virus. In segment S1 and M2, the number of nucleotide differences were high between the low and high virulent cluster (Dhamotharan et al., 2019). PRV1 isolates associated with HSMI have some unique amino acids sites in the proteins p13, σ3 and μ1. These proteins are coded by the S1 and M2 segment. These segments are genetically linked together, and this indicates that both the structure and the interactions of the encoded proteins are important for the viral fitness of PRV. This may indicate that these specific segments are important in the overall virulence of the virus isolate. However, this have been questioned in a recent paper (Wessel et al. 2020).

1.7 Aim

The main aim of this study is to identify the genotypes of PRV1 associated with heart and skeletal inflammation (HSMI) in farmed Atlantic Salmon (*Salmo salar*) in Northern Norway.

H_0 = All strains of PRV1 can cause HSMI and mortality in farmed salmon production.
H_1 = All strains causing HSMI belong to a distinct clade of PRV1.

2. Material and methods

2.1 The sampled fish

All salmon populations included in this study were reared in seawater in either Finnmark (Area A) or Nordland (Area B) (Figure 1). The salmon included in this study originates from a total of 19 sites and ~120 net pens (some sites did not include information about net pens). One of the sites (2HV) did a pre stocking sampling in freshwater before sea transfer (N = 30 smolts), and these are included in the analyzes performed in this study, but not considered in the total number of sites included in this study. Fish size in the sample set ranges from 77.4 to 4120 gram (g), with most sites sampling fish between 1000 g up until 2000 g. Genetics were from both Aquagen (**A**) and Salmobreed (**B**), with the majority of broodstock embryos originating from **A**. The smolts originates from ten different freshwater facilities (**a-j**), where **a** is the overall most used smolt provider and is present at 12 of the sites included in the study (Table 1). Sites JV, PV and KN have not provided access to their database, so most of their production data are not available (NA). Samples from these sites was provided from an external laboratory and used in both qPCR and sequencing. All the diseases listed in table 1 are diagnosed by authorized veterinarian/fish health personnel and confirmed by analyses at external laboratories.

By of the lack of any other method to evaluate the severeness and pathology of a HSMI-outbreak, the site mortalities in the category "Circulatory failure" (CF) were used. To evaluate the mortalities a limit for what is considered as elevated had to be set. In this study mortalities exceeding 2 per mil (0.02 %) in a month is considered as elevated as well as an outbreak of HSMI. This limit was selected by using the guidelines previously specified by the Norwegian Food Safety Authority. The guidelines stated that if a unit containing fish weighing more than 0.5 kg exceeds a mortality of 2.5 per mil in a day, it is considered as elevated (Akvakulturdriftsforskriften 2010, §13).

2.2 Fish sampling

The sampling performed in this project targeted salmon populations present at sea sites in the period 2019 - 2021. A total of 1475 salmon stocked at sea sites in 2018, 2019 and 2020 were sampled and included in the study. The first salmon were sampled in October 2019 and the last in January 2021. Site 2HV were the only site followed over time with eight sampling points over a period of 4 months. Samples were collected either by fish health personnel, site personnel or as fish shipped frozen with overnight courier for sampling at the University of Bergen (UoB), Fish Diseases Research Group (FDRG). Three different approaches were used for sampling of the material. Fish from sites HF and 2HV were selected randomly to provide a good as possible representative selection of the population. Fish from A2-A4 (apart from HF) were sampled independent of ongoing disease outbreak. To meet the number of samples requested, dead/moribund fish were sampled and supplemented with healthy salmons when needed. Fish from A1, B5-B8 were sampled through the monthly pathogen screening program, which requires samples from dead/moribund fish.

Table 1. Overview of the sites sampled in this study. Area A = Finnmark, Area B = Nordland. The number following the letter show the location of the site (Figure 1). The genetic origin is either capital **A** or **B**. Each freshwater site has its own designated letter from **a-j**. The date of sea transfer (**ST**), and number of net pens are included. The first month where elevated mortality (> 0.02 %) in the "circulatory failure"- category is included, as a measure of indicating when HSMI outbreak first occurred. The number of months after **ST** before the outbreak appeared is also presented. The number of day degrees at time of outbreak as well as the average temperature in degrees Celsius (° C). The month of sampling, number of fish collected (**N**) and weight in gram of fish at collection date is listed. A more comprehensive version of the table follows on page 12, where the different diagnosis, set by fish health personnel with confirmation of diagnosis from histopathological analyses conducted by an external laboratory, from each site is listed. NA = Not available.
* 2HV was sampled over time, table 11 in appendix provides more detailed information of the samplings.

Area	Site	Broodfish Embryos	Smolt	Date ST	Nets	Elevated mortality	Months after ST	Day degrees	Sample month temp.	Collection Date	N=	Weight
A1	SV	A	a	Nov/Dec-19	6	Jun-20	7	854	5.9	Nov-20	8	500
A1	EV	A	b	Oct-19	9	Jun-20	8	1066	5.6	Nov-20	6	780
A2	SN	A	ad	Apr/May-20	9	Oct-20	5	1393	8.4	Jan-21	10	1215
A2	HF	A	e	May/Jun-20	9	Sep-20	3	1077	10.2	Sep/Nov-20	120	800
A3	KO	A/B	abhj	Oct-19	8	Jun-20	8	957	7	Jul/Aug-20	98	616
A4	NN	A	a	Aug-19	9	Sep-19	1	459	8.7	Jul-20	261	220
A4	SL	A	ae	Aug-19	12	Sep-19	3	749	7.6	Jul-20	119	392
A4	KV	A	aef	Jun/Jul/Aug-19	9	Aug-19	0	222	8.1	Jul-20	77	290
A4	SH	A/B	bdeg	May/Jun-19	11	Jul-19	2	519	8.8	Aug-20	110	317
A4	OF	A	adh	May/Jun-19	11	Jul-19	1	238	5.7	Aug-20	85	200
B5	BO	A	c	Jul/Aug-19	5	NA	NA	NA	NA	Jan-20	10	NA
B5	LH	A	c	Aug-19	6	NA	NA	NA	NA	Jul-20	10	NA
B6	JV	NA	a	NA	NA	NA	NA	NA	NA	Jul-20	10	NA
B6	PV	NA	di	Aug/Sep-19	8	NA	NA	NA	NA	Mar-20	10	NA
B6	KN	NA	NA	NA	NA	NA	NA	NA	NA	Oct-19	4	NA
B7	1HV	A	ade	Aug/Sep-19	8	NA	NA	NA	NA	May-20	10	NA
B7	2HV	A/B	acei	Sep-20	8	Mar-21	6	1384	4.1	Sep-20-/Jan-21	504*	82-460
B7	HM	A	ai	Nov-19/May-20	6	APR-21	11	3044	4.7	Oct-20	6	3321
B7	VF	A	ai	Aug/Sep-19	10	NA	NA	NA	NA	Apr-20	6	NA
B8	HO	A	abc	Apr/May/Jun-20	11	Nov-20	6	2115	8.2	Oct-20	10	1830

Continuing of Table 1 Area, site, and diagnosis from each site where the HSMI diagnosis is emphasized.

Area	Site	Diagnosis
A1	SV	Ulcerative disease, parvicapsulosis, **HSMI**
A1	EV	Nefrocalsinosis, ulcerative disease, parvicapsulosis, **HSMI**
A2	SN	Desmoltification, production disorders, ulcerative disease, **HSMI**
A2	HF	Production disorders, ulcerative disease, **HSMI**
A3	KO	Nefrocalsinosis, ulcerative disease, parvicapsulosis, **HSMI**
A4	NN	Parvicapsulosis, winter ulcers, **HSMI**
A4	SL	Parvicapsulosis, **HSMI**
A4	KV	Epitheliocystis, parvicapsulosis, **HSMI**
A4	SH	Nefrocalsinosis, winter ulcers, parvocapsulosis, IPN, **HSMI**
A4	OF	Nefrocalsinosis, parvocapsulosis, **HSMI**
B5	BO	Ulcerative disease, **HSMI**
B5	LH	Tenacibaculosis
B6	JV	Tenacibaculosis, nefrocalsinosis, SGPV, **HSMI**
B6	PV	NA
B6	KN	**HSMI**
B7	1HV	Proliferative gill disease, ulcerative disease
B7	2HV	Ulcerative disease
B7	HM	Ulcerative disease, **HSMI**
B7	VF	Tenacibaculosis, parvicapsulosis, **HSMI**
B8	HO	Tenacibaculosis, **HSMI**

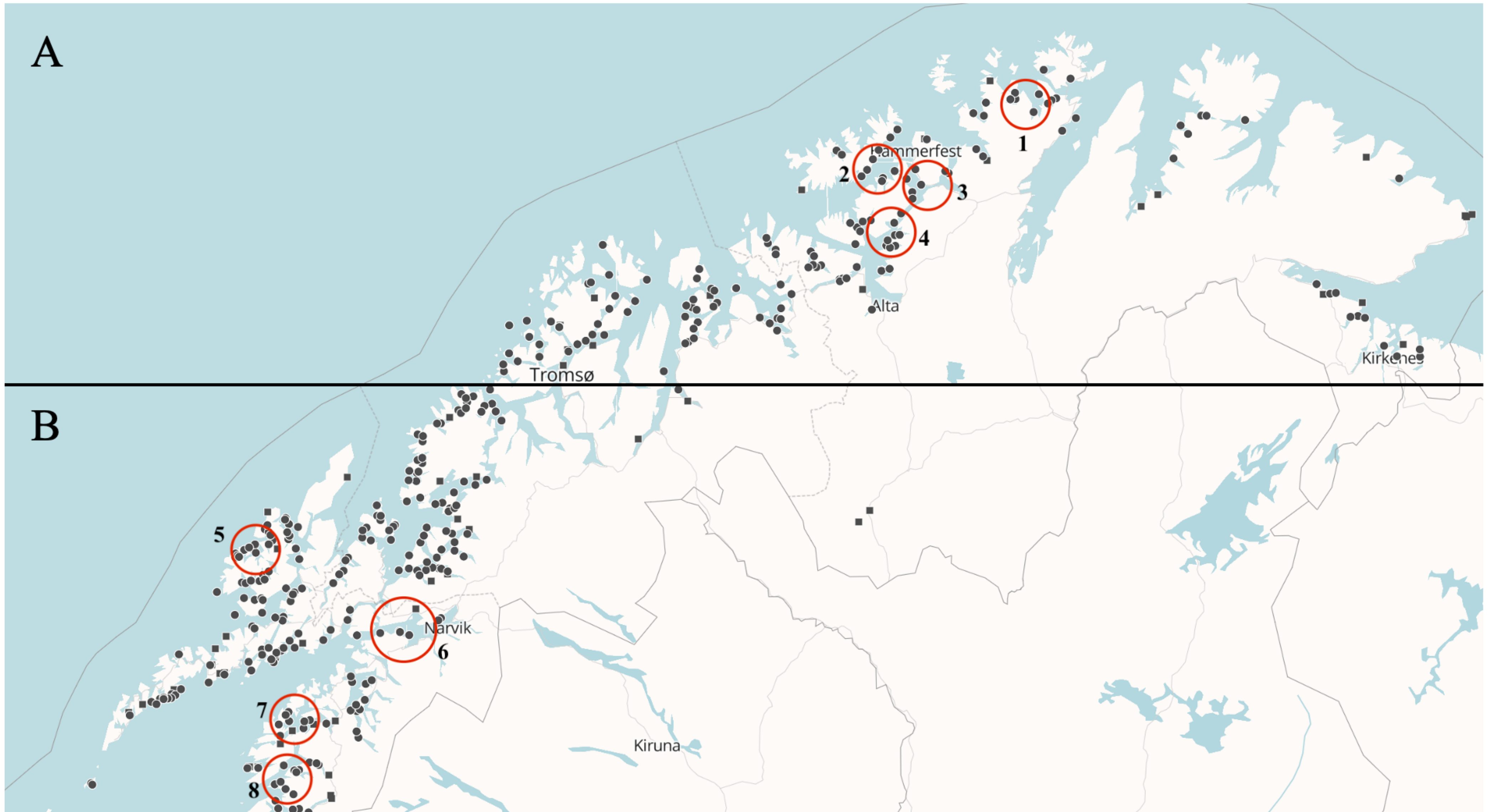


Figure 1 Map showing the geographical location of the sampling sites distributed in either Area **A** or Area **B**. 1 = SV, EV. 2 = SN, HF. 3 = KO. 4 = NN, SL, KV, OF, SH. 5 = LH, BO. 6 =JV, PV, KN. 7 = HM, VF, HV. 8 = HO.

2.3 Processing of samples

Processing of samples were performed using three different protocols:

1. Sampled tissues at sites in Area A1-4 (apart from HF) (total of 750 salmon) were fixated in RNAlater before being shipped to an external laboratory (Pharmaq Analytic) for RNA extraction.

2. Using the pathogen screening program of the salmon producer, historical positive PRV samples were identified in an existing biobank at the screening laboratory. This applies to sites in Area B5-8 (apart from 2HV) (total of 100 salmon). Tissue samples fixated in tubes with RNAlater were shipped to the UoB facilities from PatoGen AS. The tissues were cut to an appropriate size and transferred to a 1.5 mL tube before continuing the RNA extraction (2.3).

3. At site HF and 2HV (total of 625 salmon) the salmon was shipped with overnight carrier to the UoB. The sampled tissues were put on ice and frozen until the date of RNA extraction.

2.4 RNA-extraction

For those samples in the project from which RNA was extracted at the UoB, the manufactures protocol (TRIzol® Reagent, by life technologies, Invitrogen) was followed with some modifications.

1000 µL of TRI reagent was added to the tissue and homogenized for 3 minutes at 30.0 Hz (TissueLyser II Qiagen). All tissue except gill were homogenized using a sterile steel bead.
The samples were incubated at room temperature for 5 minutes. 200 µL of chloroform was added, mixed by manual shaking for 20 seconds, followed by 5 minutes of incubation at room temperature. The tubes were centrifuged at 4°C using 12 000 x g for 15 minutes. 400 µL of the aqueous top layer was collected and transferred to a new tube containing 500µL isopropanol, then turned upside down 2 times to mix. The tubes were incubated for 10 minutes at room temperature, centrifuged at 4°C using 12 000 x g for 15 minutes, resulting in a white pellet of RNA at the bottom side of the tube. The supernatant was discarded and 1000 µL of 75 % ethanol was added, followed by vortexing and centrifugation at 12 000 x g at 4 °C for 5 minutes before discarding the ethanol. This procedure was repeated using 100 % ethanol. The tubes were left open to air-dry the pellet 10-15 minutes to let the alcohol evaporate.

The pellet was resuspended in 200 µL nuclease-free water pre-heated to 70 °C. After extraction, the RNA samples were stored at -80 °C. Negative controls without tissue were included in each RNA extractions and followed the same protocol as samples with tissues.

2.5 Real time RT-PCR

The extracted RNA was analyzed by Real time RT-qPCR for detection of target RNA. qPCR was performed using the AgPath-ID^{TM} One-Step RT-PCR Kit (Applied Biosystems) with some modification of the manufactures protocol. The RNA was analyzed using the following assays; EF1A, targeting the Elongation factor 1 alpha (EF1A) of *Salmo salar*. PRV1 targeting the PRV1 M2-gene. PRV1-A3 targeting the PRV1 S1-gene (Table 2).

Table 2. List of the different targets, primers, and probes for the selected selections in RT-qPCR with the associated sequence.

Target	Primer/Probe	Direction	Sequence (5-3')	Reference
Piscine orthoreovirus 1	PRV1-M2-F	Forward	CAATCGCAAGGTCTGATGCA	(Are Nylund et al., 2018)
	PRV1-M2-R	Reverse	GGGTTCTGTGCTGGAGATGAG	
	PRV1-M2-probe	Probe	CTGGCTCAACTCTC	
Piscine orthoreovirus 1b	PRV1-A3-F	Forward	GGCAUCGGUUGGUUUGUC	(Siah et al., 2020)
	PRV1-A3-R	Reverse	UCAUCAAUGCGACGGCG	
	PRV1-A3-probe	Probe	AUAAUGCUAAGCCUGUUAUGGU	
Salmon elongation factor 1 Alpha A	EF1A F	Forward	CCCCTCCAGGACGTTTACAAA	(Olsvik et al., 2005)
	EF1A R	Reverse	CACACGGCCCACAGGTACA	
	EF1A P	Probe	ATCGGTGGTATTGGAAC	

Master mix and template were added to MicroAmp® Optical 96-well Reaction Plates (Applied Biosystems). Plates were kept cooled prior to analysis. The total volume in each well was 12.5 µL using 10.5 µL master mix and 2 µL template. The Master mix consisted of 6.25 µL 2X RT-PCR Buffer, 1.0 µL forward and reverse primer (800 nM), 0.22 µL TaqMan® Probe (175 nM) and 1.78 µL nuclease-free water. The Master mix for the PRV1-A3 assay was adjusted, as described in table 3.

Table 3. Volume of each of the reagents of the Master mix for PRV1-A3 assay including the concentrations of the probe, forward- and reverse primers.

Reagent	PRV1-A3 assay
2X RT-PCR Buffer	6.25 µL
Forward primer (900 nM)	1.13 µL
Reverse primer (600 nM)	0.75 µL
TaqMan® Probe (225 nM)	0.28 µL
25X RT-PCR Enzyme mix	0.5 µL
Nuclease-free water	1.59 µL

For each qPCR run, a negative RNA-extraction control (NC) and a non-template control (NTC) were included. NC were primarily included to detect any potential contamination of RNA during the extraction and NTC were included to reveal contaminations of the Master mix. The plates were sealed with MicroAmp^{TM} Optical Adhesive Film (Applied Biosystems (Thermo Fischer Scientific)), centrifuged, and analyzed in an Applied Biosystems® QuantStudio 3 Real-Time PCR Systems. All reactions were run as default AgPath setup, reverse transcriptions for 10 minutes at 45 ºC, denaturation and activation of the DNA polymerase for 10 minutes at 95 ºC. The run was extended to 45 cycles of amplification with denaturation at 95 ºC for 15 seconds and then amplification at 60 ºC for 45 seconds. Samples giving a Ct-value < 40.0 were considered positive.

2.6 cDNA synthesis

Sequencing was performed on PRV-positive qPCR samples with Ct-value < 25.0. 2-3 samples were selected from each of the 19 sites. At sites where the qPCR screening indicated the presence of PRV1a, a selection of these samples was included for sequencing. cDNA synthesis was performed on all the samples selected for sequencing, using the RevertAid First Strand cDNA Synthesis (ThermoFisher Scientific). The primer chosen for the synthesis was the random hexamer primer. For each reaction 0.5 µL Random Hexamer Primer (100 ng/µL), 3.5 µL nuclease-free water and 2 µL of RNA-template was added to a 0.2 mL tube before the samples were put in an Applied BiosystemsVereti 96 well Thermal cycle for 5 minutes at 65 ºC. Then 6 µL of a solution consisting of 2 µL 5x Reaction buffer, 0.5 µL RiboLock, 1 µL of dNTPs and 0.5 µL MMLV was added, and the samples were run at 25 ºC for 5 min, 42 ºC for 60 min and 70 ºC for 5 min. The cDNA was diluted 1:4 and frozen at -20 ºC until used.

2.7 Primer design

Initial testing of available in-house PCR and sequencing primers targeting M2-gene of PRV1 were shown to be inadequate to amplify larger parts of the gene. New primers where therefore designed for this project aiming to amplify most of the M2 gene. The primer sets were designed using known M2-sequence from GenBank using the software Vector NTI. The primers are shown in Table 4.

2.8 PCR

PCR was performed using the primers listed in table 4. The master mix used in each reaction contained 17.7 µL nuclease-free water, 0.2 µL MgCl, 2.5 µL buffer, 0.4 µL dNTPs, 1 µL forward and reverse primer and 0.2 µL Taq DNA Polymerase. Then 2 µL template was added to each tube.

The samples were run in an Applied BiosystemsVereti 96 well Thermal cycler. The following PCR cycle was used: denaturation for 2 minutes at 95 ºC, then denaturation for 30 seconds at 95 ºC, annealing for 1 minute at 55 ºC and elongation for 30 seconds at 72 ºC, and this is repeated for 35 cycles. Lastly the extension for 5 minutes at 72 ºC.

Table 4. Overview over PCR and sequencing primers used to target the S1 and M2 gene of *Piscine orthoreovirus*.

Target	Primer	Direction	Sequence (5-3')	Reference
S1 gene	PRV1-S1F1	Forward	GATAAAGACTTCTGTACGTGAAAC	Present study
	PRV1-S1R2	Reverse	TGCTCCACTGGGTTCAGCTC	
S1 gene	PRV1-S1F2	Forward	AAACCCAAATGGCGAACCA	Present study
	PRV1-S1R3	Reverse	ACAGTAGGCTCCCCATCACG	
M2 gene	PRV1-M2F1	Forward	AATTTGTTTAACAGGCTTGACC	Present study
	PRV1-M2R1	Reverse	GATTGGAGTTGAATGAGGGA	
M2 gene	PRV1-M2F2	Forward	TCTGAGAGAACTGAGAAGCC	Present study
	PRV1-M2R2	Reverse	CGRCCACTGTCAGTRAATTG	
M2 gene	PRV1-M2F3	Forward	CCTCACCACRCCCTGGGTAT	Present study
	PRV1-M2R3	Reverse	TCTGTCAACCTCAACYCCTT	
M2 gene	PRV1-M2F4	Forward	AATCTCTCTGCATCCACCAC	Present study
	PRV1-M2R4	Reverse	TGGTGGAAACAGTTTCTCTA	

To visualize the present strands of DNA in the amplified PCR product gel electrophoresis was conducted. The gel is made by 1 % agarose solved in 1X Tris-acetate (TAE) buffer and added 1 µL of fluorescent dye GelRed™ pr. 25 mL agarose solution to stain the nucleic acids. The gel solidified in 10 minutes, then covered in 1X TAE buffer. The first well was filled with 2.5 µL GeneRuler 100 bp Plus DNA ladder (ThermoFischer Scientific) as a molecular weight marker, 5 µL PCR product was mixed with 1 µL loading dye (6x TriTrack DNA Loading Dye (T) and 5 µL was added to each well in the gel. The gel was run at 80 Volts for 40 minutes and examined by UV-light (Carestream GelLogic 212 Pro) in the Carestream MI (v.5.0.2.30) program.

PCR products, evaluated on the gel, as sufficiently amplified (as single strong bands at appropriate size) was purified using ExoSAP-IT (Applied Biosystems™) prior to being used as template in the sequencing reaction. ExoSAP-IT cleans the product by enzymatic degradations of primers and dNTP's to prevent interfering of the sequencing process. 2.5 µL PCR product was added in a tube containing 1µL ExoSAP-IT and run in the PCR machine at the standard program recommended in the ExoSAP-IT kit: 15-minute incubation at 37 ºC to remove nucleotides and primers followed by 15 min at 80 ºC to inactivate present enzymes. PCR-

products showing presenting as strongly fluorescent in the gel-electrophoresis, indicating high amount of amplicon, was diluted 1:5 by adding 14 µL nuclease free water after purifying.

2.9 Sequencing

Sangers sequencing using Big Dye chemistry was performed to identify the PRV1 genotype in the sampled salmon. 1 µL purified PCR-product (template) was added in 0.2 ml PCR-tube containing: 1 µL BigDye, 1 µL Buffer, 6 µL nuclease-free water and 1 µL of forward or reverse primer. All sequencing was performed in both directions. The primers used for sequencing are the same as for acquiring the PCR amplicon used as template (Table 4). The BigDye cycle was run in an Applied BiosystemsVereti 96 well Thermal cycler using an initial single denaturation for 5 minutes at 96 ºC, followed by 25 cycles of denaturation at 96 ºC for 10 seconds, annealing at 50 ºC for 5 seconds and elongation at 60 ºC for 4 minutes. 10 µL nuclease-free water was added before the final sequencing at seqlab at UiB (https://www.uib.no/en/seqlab).

2.10 Phylogenetic analyses

The nucleotide sequences of PRV1 segments M2 and S1 were assembled with the help of Vector NTI software (InforMax, Inc.). GenBank searches were done with blastn (2.7.1). The Vector NTI Suite software package (InforMax, Inc.) was used for the multiple alignments of the segment sequences. To perform pairwise comparisons the multiple sequence alignment editor GeneDoc (Available at: www.psc.edu/biomed/genedoc) was used for manual adjustments. A selection of sequences available from the EMBL nucleotide database were included for comparisons (Table 5). The phylogenetic trees were obtained by analysis of 1951 nt from segment M2 (Position 27 – 1977 in the ORF, Accession no: GU994016) and 960 nt from S1. (Position 34 – 993, Accession no: GU994022). These trees were constructed using TREE-PUZZLE 5.2 (Available at: http://www.tree-puzzle.de), maximum likelihood (ML). The maximum likelihood trees were bootstrapped (50000 puzzling steps) in TREE_PUZZLE. The PRV1 from the Faeroe Islands (Accession nos: MK675876 and MK675888) were used as outgroup for the analysis of M2 and S1, respectively. Phylogenetic trees were drawn using TreeView (Page, 1996).

Table 5. Overview of PRV1 sequences of segments M2 and S1 obtained from the GenBank. N = Norway, F = Faeroe Islands, Can-BC = Canada – British Colombia, Chile, and USA.

Country	Year	PRV1	M2	S1
N	1988	NOR-1988	MK675866	MK675868
N	1996	NOR-1996	MW279855	MW279857
N	1997	NOR-1997	MK675826	MK675828
N	2005	NOR-2005/TT	MK675836	MK675838
N	2007	50607 (2007)	KR337477	KR337479
N	2010	GP-2010/NOR	GU994016	GU994022
Chile	2011	CGA280-05	KC795569	KC795571
Can-BC	2011	A.3.2-36-G609	MH093952	MH093978
Can-BC	2011	A.3.2-69-G531	MH093953	MH093979
Can-BC	2011	P.2-1_G577	MH093958	MH093984
Can-BC	2012	VT06062012-358	KC715683	KC473453
Can-BC	2012	VT06202012-371	KC776260	KC473454
Can-BC	2012	P.3-3_G729	MH093962	MH093988
Can-BC	2012	P.3-120_G417	MH093964	MH093990
N	2012	NOR2012-V3621	KY429947	KY429949
Can-BC	2013	BCJ19943-13	KT429734	KT429736
Can-BC	2013	BCJ31915-13	KT429744	KT429746
Can-BC	2013	B7274	KX851974	KX851971
Can-BC	2013	B5690	KX851975	KX851970
Can-BC	2013	A.3.2-153_G808	MH093954	MH093980
Can-BC	2013	A.3.5-168_G860	MH093957	MH093983
Can-BC	2013	P.2-3_G460	MH093959	MH093985
Can-BC	2013	P.3-37_G446	MH093963	MH093989
USA	2014	WSKFH12-14	KT429754	KT429756
N	2015	NOR-2015/MS	MK675846	MK675848
N	2015	NOR-2015/SSK	MK675856	MK675858
F	2015	FO/1978/15	MK675876	MK675878
Can-BC	2016	16-005	MH347363	MH347365
Can-BC	2016	16-011	MH347373	MH347375
F	2016	FO/41/16	MK675886	MK675888
N	2018	Nor-2018/SF	MW260139	MW260141
N	2018	Nor-2018/NL	MW260149	MW260151
Can-BC	2018	CAN-16-005ND-V4105	MW279875	MW279877

3. Results

3.1 Production results

Data from production was registered into a collective database (Fishtalk). This database holds registered information on relevant production data such as mortalities, genetic origin, freshwater sites, weight, temperature, and number of day degrees. Data was gathered and registered by site personnel. Limited data were available for JV, PV and KN. All mortalities throughout the production period were registered on cause. In the present study the mortality caused by “Circulatory failure” (CF) was used to evaluate HSMI mortality. There are two main causes to CF in production region A and B, CMS and HSMI. No CMS was diagnosed on any of the sites in the study and piscine myocarditis virus (PMCV) was only detected on two sites (SN and KO). This in contrast to PRV1 and HSMI, which was present on most sites. It was therefore considered that the CF category would be representative for the mortality caused by HSMI for the sites included in this study. Data for CF was used to describe the HSMI outbreaks and their severity.

Production of the salmon was performed in the Arctic region of Norway; this is reflected in the temperature records which typically show a temperature range of 1.9 °C – 12.1 °C in Area A and 3.1 °C – 14.1 °C in Area B. Average mortality after 6, 12 and 18 months of production was 0.10 %, 0.44 % and 0.87 % on site level. The most prevalent diagnoses besides from HSMI were ulcerative disease, parvicapsulosis and nefrocalsinosis. A typical HSMI outbreak in the dataset lasted for 9 months. The total loss due to circulatory failure on site level varied between 0.0 % to 3.6 % and the average accumulated loss at all sites were 1.0 %. Reports on diagnoses performed by authorized fish health personnel was used to provide info about diseases at the sites. The first month with elevated mortalities caused by circulatory failure is used as a base for calculating when an outbreak occurred.

3.2 Site mortality

Site mortalities in CF category was used as a tool to evaluate if any outbreaks of HSMI occurred during the production cycle. HSMI will normally cause elevated mortality over a longer time span. Not all sites in the study completed their production cycle prior to the collection of data were in May 2021. Most sites were either harvested or in last period of production. The mortalities varied from facility to facility, as well as within the units in a facility (Figure 2-5). One of the sites, VF, did not experience any elevated mortality through the production cycle. The other sites had at least 1 outbreak of HSMI through the production. The sites JV, PV or KN is not included because of the lack of access to their database. No data exists in the CF category for sites BO, LH and HV as they did not use this category in their database when classing mortality causes.

The average accumulated mortality based on the CF category was 1.0 % of the total mortality when comparing all sites included in this study. Only two sites exceed 2.0 % mortality, one with a mortality of 2.5 % (KV) and the other with a mortality of 3.6 % (KO). The latter site was the most affected site in this study. The most affected net pen had a total mortality of 9.3 % at harvest. The total mortality of each of the included sites can be seen in Figure 6.

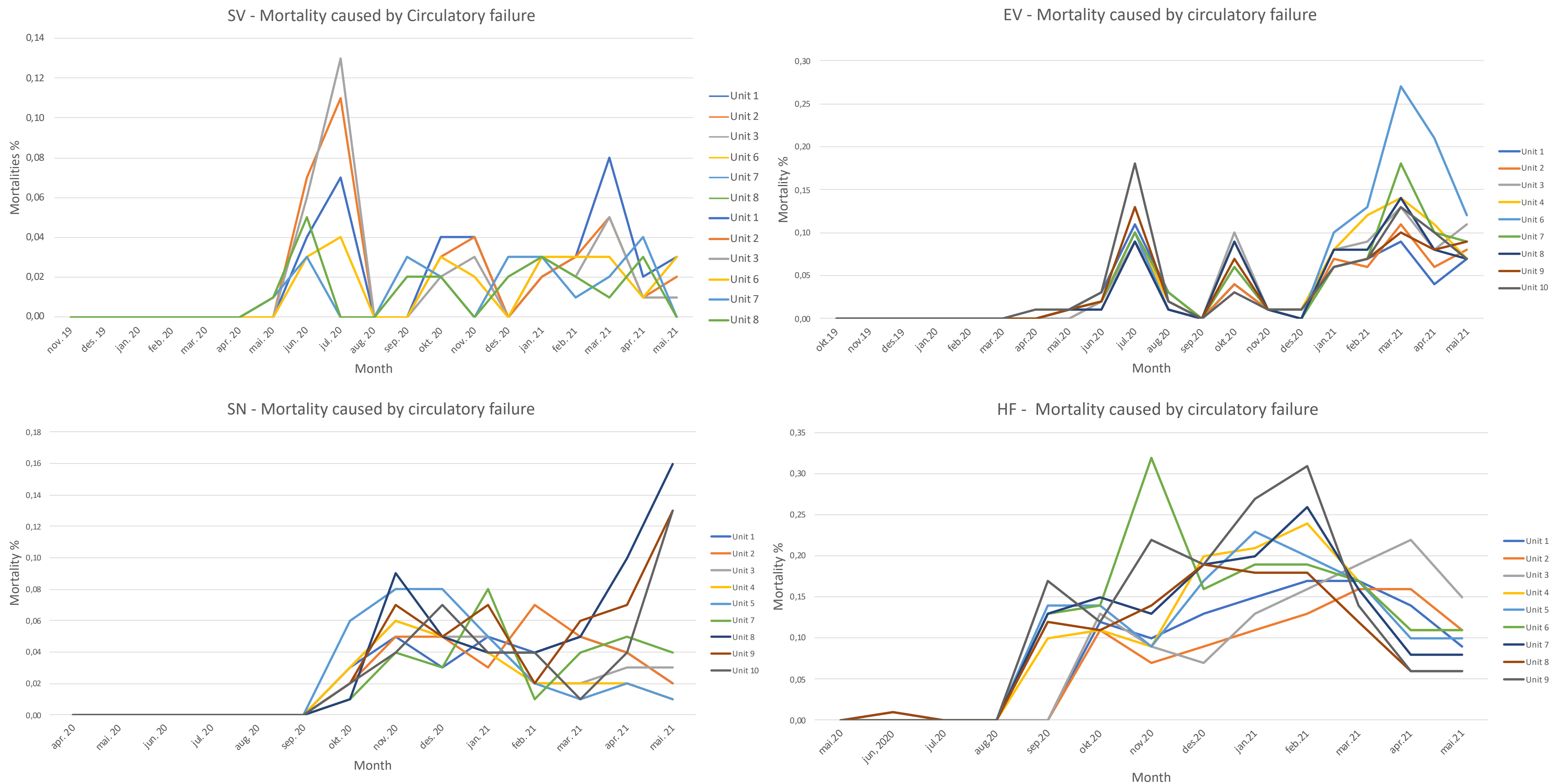


Figure 2 Graphs showing the monthly mortality caused by circulatory failure at netpen level for the sites SV, EV, SN and HF. All sites are located in Area A. The number of months after sea transfer is illustrated at the x-axis. The y-axis illustrates mortalities in %. It varies from site to site when the mortalities start after ST. SV have one outbreak only lasting for 2 months, before a new elevation in mortalities that last throughout the time of the study period. Site EV have a distinct pattern to the elevating in mortalities, which is showing several outbreaks of HSMI. Both SN and HF have one single outbreak that stays elevated through the study.

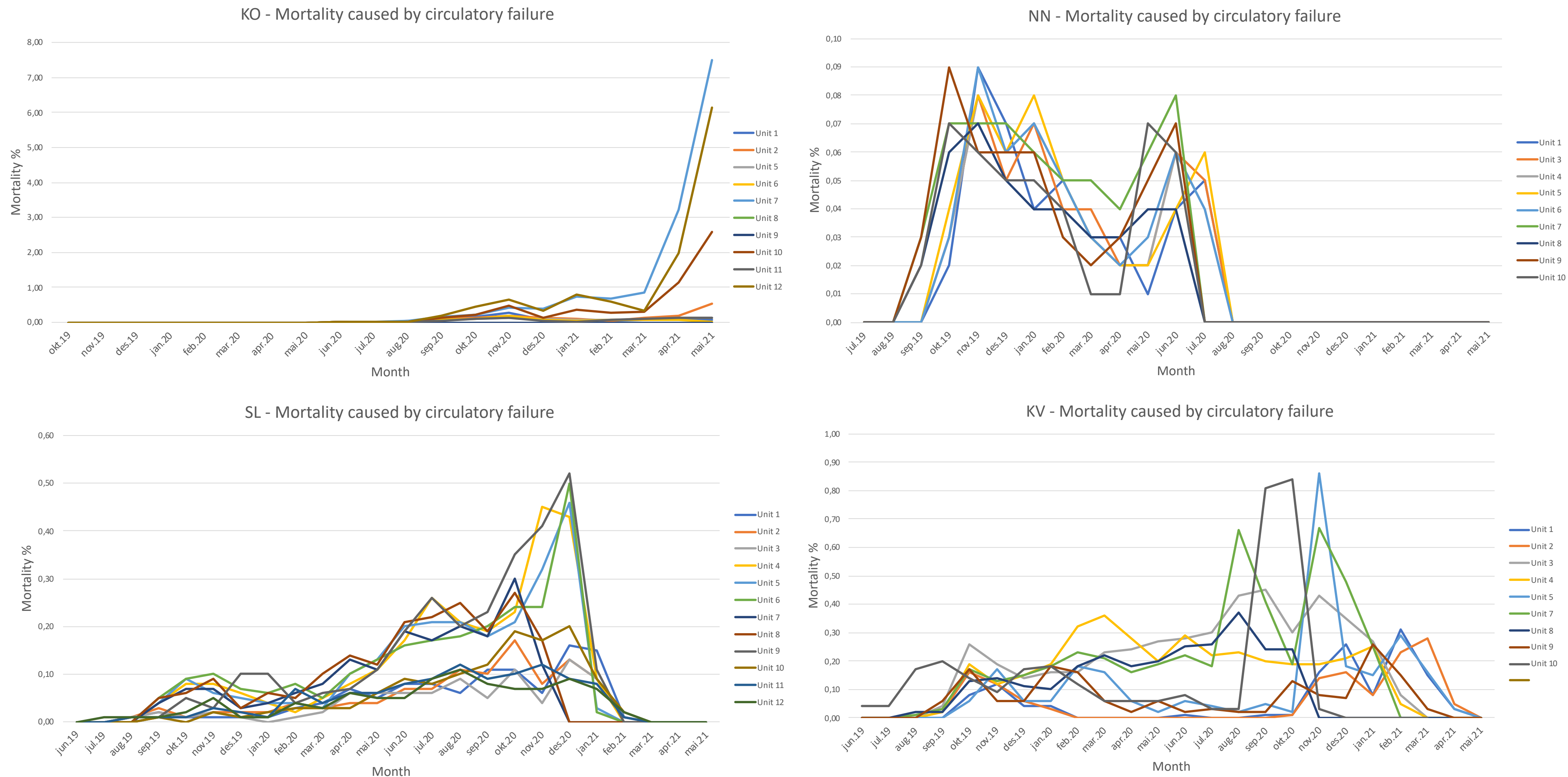


Figure 3 Graphs showing the monthly mortality caused by circulatory failure at netpen level for the sites KO, NN, SL and KV. All sites are located in Area A. The number of months after sea transfer is illustrated at the x-axis. The y-axis illustrates mortalities in %. Site KO experience elevated mortalities in June 2020 before the mortalities declines. Then they elevate again and stays high. Unit 7 is the most affected net pen with a mortality of 7.5 % in May 2021, and a total accumulated mortality of 9.3 %. At NN the mortality is elevating 1 month after sea transfer and stays elevated until harvesting. The mortalities at SL and KV is elevated throughout the production as well. The mortalities at KV stays elevated for 22 months, which is the longest consistent mortality throughout this study.

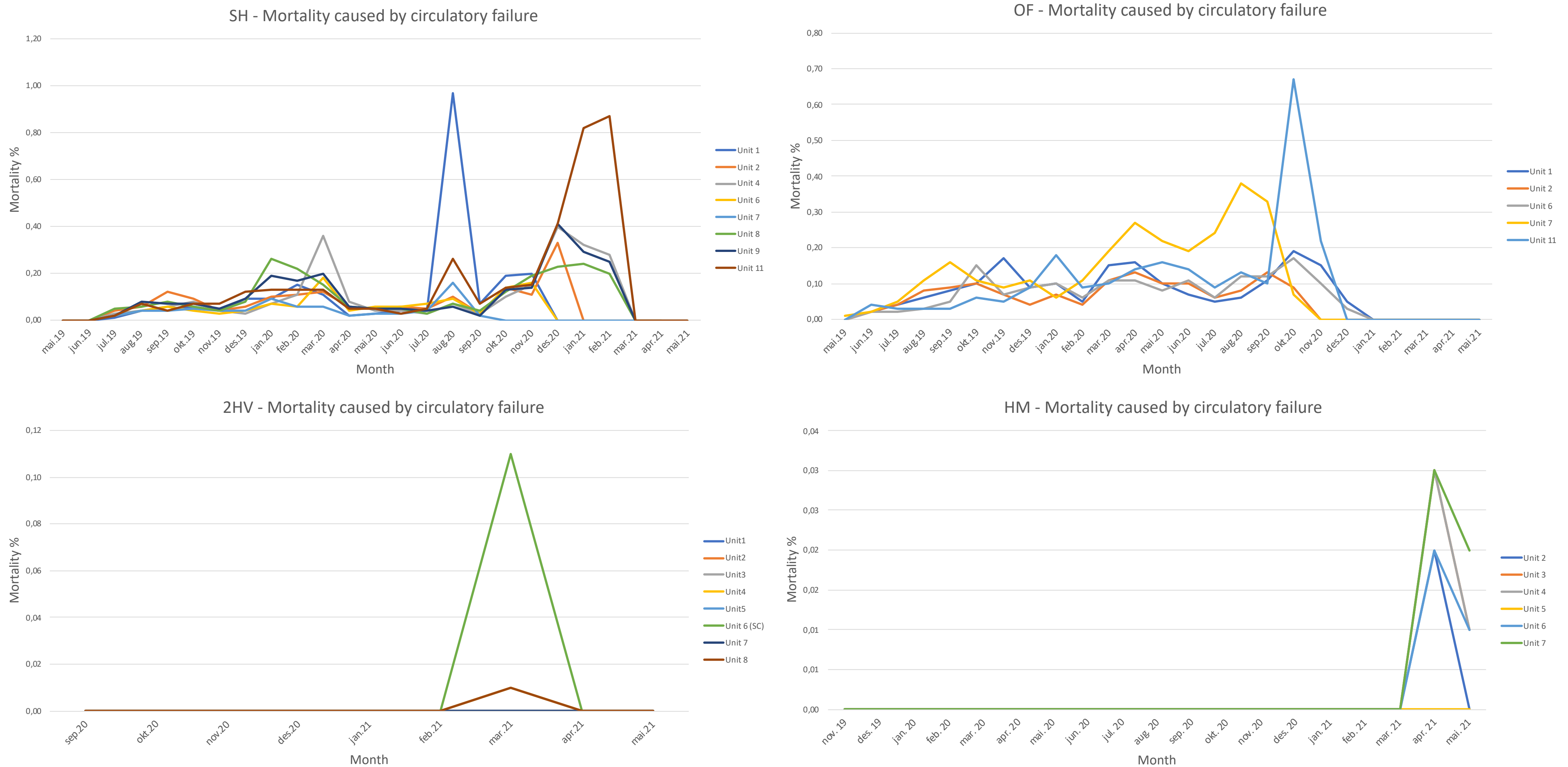
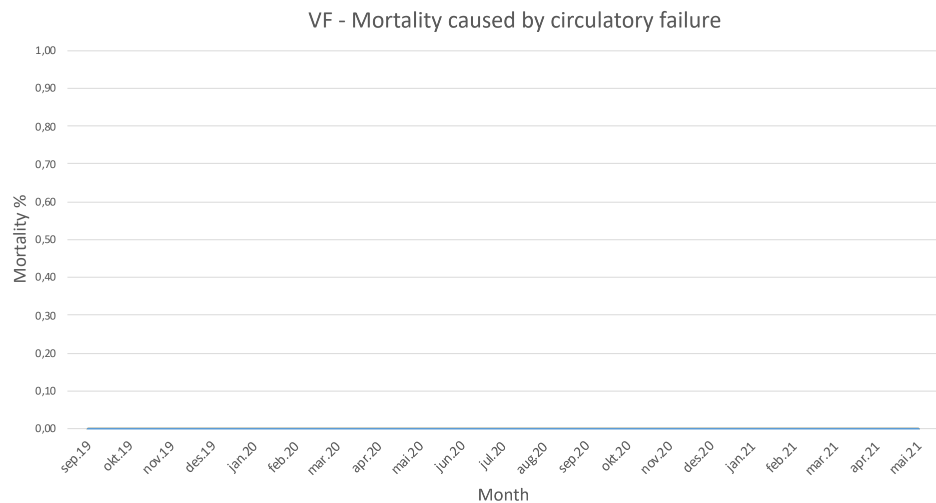


Figure 4 Graphs showing the monthly mortality caused by circulatory failure at netpen level for the sites SH, OF, 2HV and HM. SH and OF are located in Area A, 2HV and HM are located in Area B. The number of months after sea transfer is illustrated at the x-axis. The y-axis illustrates mortalities in %. SH and OF have an HSMI outbreak that continuous throughout the production cycle, and they last for 19-20 months. The mortality is fluctuating. 2HV have an outbreak of HSMI 6 months after sea transfer, which decreases after a month. HM have a late outbreak of HSMI, and because of the harvest it only lasts for 2 months.



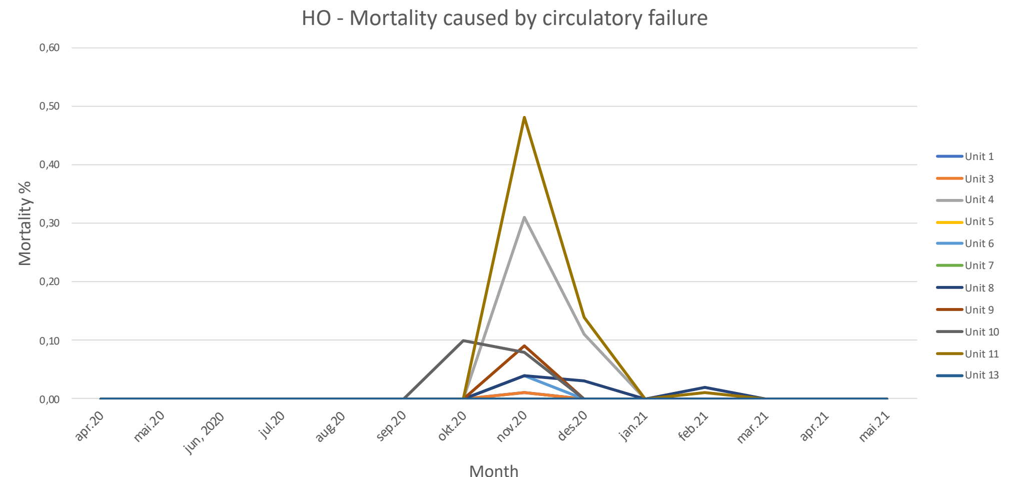


Figure 5 Graphs showing the monthly mortality caused by circulatory failure at netpen level for the sites VF and HO. Both sites are located in Area B. Graphs showing the mortalities caused by circulatory failure. The number of months after sea transfer is illustrated at the x-axis. The y-axis illustrates mortalities in %. Both sites are found in Area B. VF did not have any outbreaks of HSMI throughout the production cycle. HO had an outbreak lasting for 3 months until the mortalities decreased again.

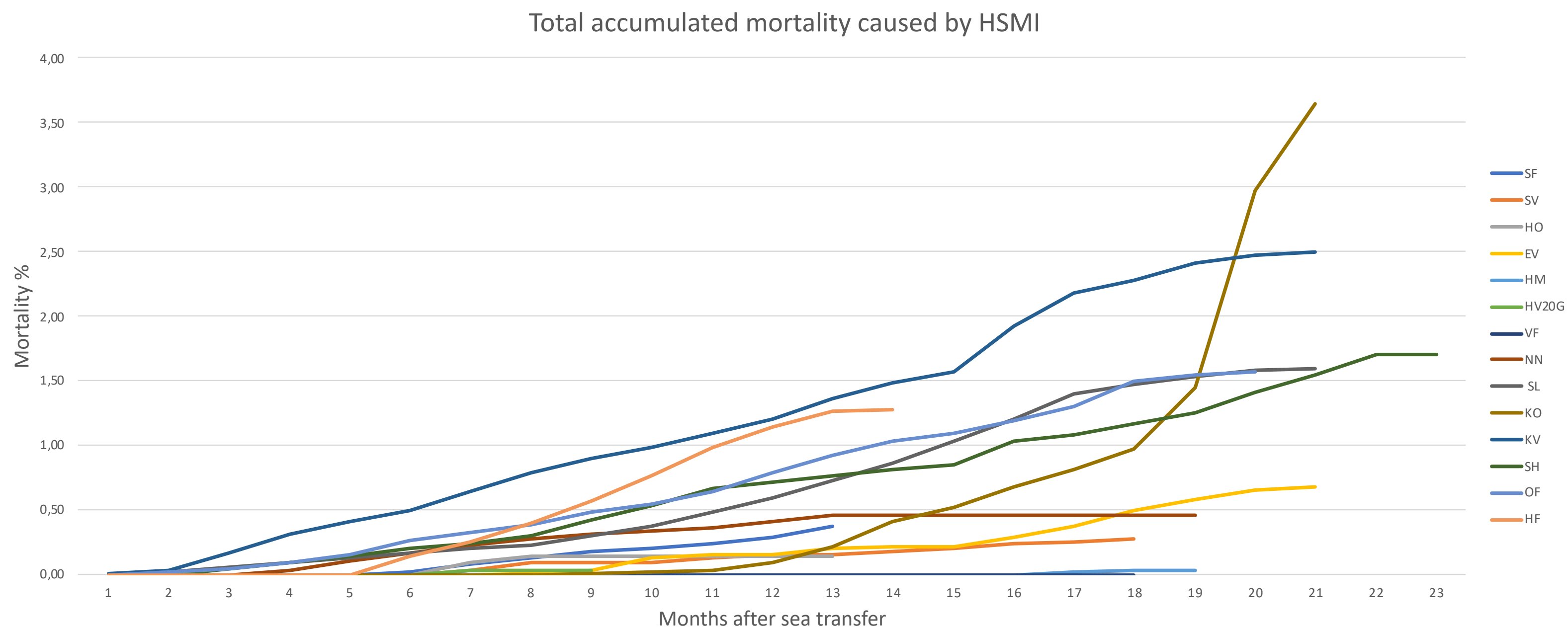


Figure 6 Graph showing the total accumulated mortality by HSMI. The number of months after sea transfer is illustrated at the x-axis. The y-axis illustrates mortalities in %. One site had 0 % mortality (VF) and the most affected site had 3,69% mortality (KO). The graph shows the variation in production cycles, where some sites have a cycle of 21-23 months and others have a cycle of 18-19 months. Three of the sites (HF, KV and HO) did not finish the production cycle during this study. PV, JV and KN is not included in the graph.

3.3 Outbreaks of HSMI

Both the occurrence of the first HSMI and the duration of the outbreak varied between both the Areas and the sites. Six of the sites experienced the first outbreak between 0-3 months after sea transfer, which is nearly half of the sampled sites in this study. The duration of these outbreaks lasted from 1 to 3 months. Only three of these sites experienced another outbreak of HSMI during the production cycle (SV, EV, KO), which lasted for 5-10 months. Most of the production cycles in Area A is affected by HSMI for a longer period of time. Sites that completed the production cycle during this study experienced elevated mortalities for a minimum of 11 months and up until 22 months. The two remaining sites have experienced continuous mortalities for 6 and 9 months of the production so far. The production data from Area B only covers four of the sampled sites. The duration of HSMI only lasted between 0-3 months, and one of the sites only experienced elevated mortality late in the production cycle (HM). One of the sites did not experience any disease outbreak at all. Figure 7 shows both A) when the first outbreak of HSMI occurred and B) the duration of this outbreak.

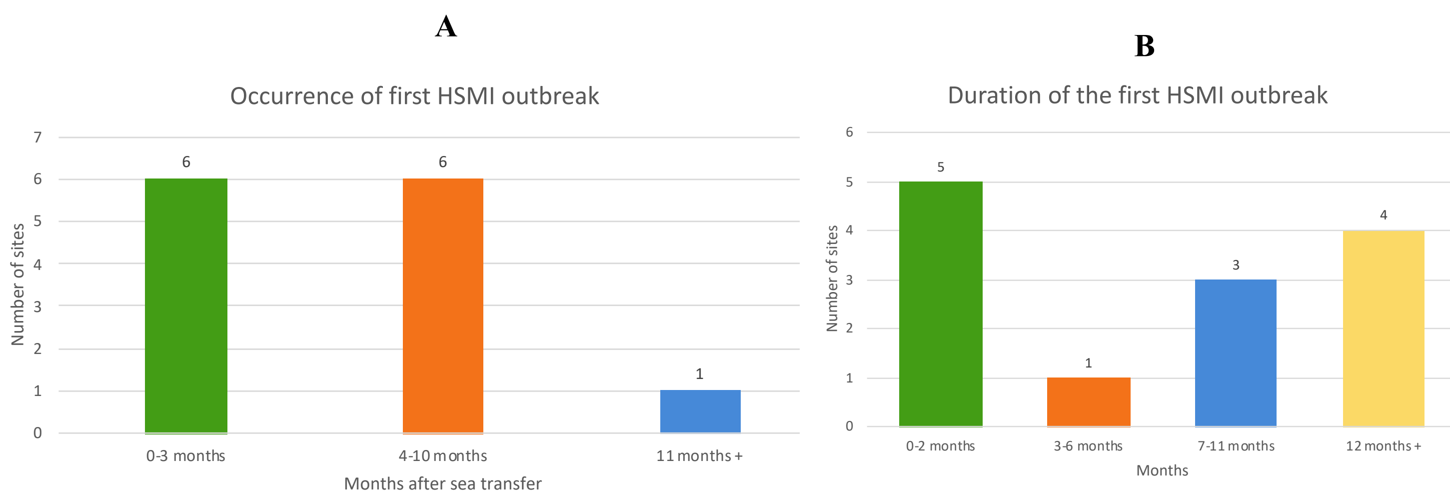


Figure 7. A) Bar charts illustrating when sites experienced the first outbreak of HSMI after sea transfer (left) and **B)** the duration of the outbreak (right). The x-axis at **A** illustrates months after sea transfer while the x-axis at **B** illustrates number of months. The y-axis illustrates number of sites.

3.4 Real time RT-PCR

qPCR was used to quantify the levels of PRV1 RNA in all samples. RNA extracted from tissue were analyzed using a universal PRV1 assay targeting M2 to detect the presence of PRV1. The lower the Ct-value the more of the virus is present in the sample. All tissue found positive with the universal PRV1 assay were re-tested using an assay (PRV1-A3) specific for members of clade PRV1b. The PRV1-A3 assay targets S1 segment in the PRV1b genogroup. This segment is considered a putative virulent marker and the assay was made to target the putative high virulent and HSMI causing genogroup. The qPCR results from these to assays were then used to predict which of the genogroup the isolate belonged. If a sample was found positive with the universal PRV1 M2 assay but negative with the PRV1b specific PRV1-A3 assay, the sample was assumed to be infected with the putative low-virulent variant, PRV1a. This is the first big-scale screening with the PRV1-A3 assay. It was tested on 752 samples, where a total of 25 samples was 1b-negative. 4 PRV1b-negative samples were selected, based on low Ct-values, for sequenced (SL-1, KO-3, OF-10, NN-12).

All the sites included in this study were positive for PRV1. The lowest obtained Ct-value was 12.8. The highest registered Ct-value was 40.0. The Ct-values varied between the sites. Some sites had little variation between the lowest and highest obtained Ct-value, such as values between 15.0 - 17.9 (HV), while other had a larger span between the values, such as 17.7 and up to 40.0 (HF).

All obtained Ct-values from each of the sites can be found in table 12 - 39 in appendix.

3.5 Prevalence of PRV1

The prevalence of PRV1 varied from site to site. In Finnmark (Area A) the prevalence varied a lot more than in Nordland (Area B) (Table 6). For sites with several sampling points, the prevalence was calculated using the last sampling at the sites (HF, 2HV). The sites in Area A had a prevalence of PRV1 between 52.2 % up to 100.0 %. At six of the ten sites, the PRV1a-variant of the virus was identified through qPCR. The site SL had a prevalence of 10.5 % when looking at PRV1a, which is the highest prevalence of this genogroup found in this study. When comparing the prevalence of PRV1a and PRV1b, the latter is by far the dominating variant, and it is present at all screened sites in both areas. At 2HV and HF the sampling was performed with an aim to obtain a good representation of the population by sampling only seemingly healthy salmon. This, combined with the limited number of months spent in the sea did probably contribute to the low prevalence of the virus. Most of the Ct-values were high. HF was sampled both in September (N = 30) and November (N = 90) in 2020. At first sampling the fish had been in the sea for 4 months, and 6 months in the second sampling. Only three of the fish sampled in September were positive for PRV1 where one had a Ct-value of 20.9, and the two other had a Ct-value: > 35.0. In the following sampling 41 were positive for PRV1. 31 of these had a Ct-value > 30, while the remaining 10 had a Ct-value of < 25.0. The increase in prevalence (nearly 40 %) and increase in mortalities when the second sampling were conducted indicate that the fish were in a viremic phase of the infection. The screening results from 2HV is found in Table 7. None of the screened samples were identified as PRV1a, and the site did not enter a viremic phase during the time of this study.

The high prevalence and the low Ct-values obtained from salmon in Area A1 and B5-8 (Apart from 2HV), was expected because of the approach in obtaining these samples. These samples were collected during a monthly pathogen screening program (MSP) and the PRV1-positive samples was deliberately chosen for this project. This to ensure samples with high amounts of virus that could be easily sequenced. Only samples with a Ct-value > 25.0 were sequenced.

Table 6. Overview of the prevalence from each of the sites included in this study, geographical area (Figure 1). The sampling methods column show sampling strategy applied at the site, MPS (Monthly pathogen screening), SR (Screening independent of disease outbreaks) and R (Randomized). The table also provide info of the obtained prevalence at each site, both for PRV1b and PRV1a.

Area	Site	Sampling method	Prevalence PRV1b	Prevalence PRV1a	Area	Site	Sampling method	Prevalence PRV1b	Prevalence PRV1a
A1	SV	MPS	100.0 %	-	B5	BO	MPS	100.0 %	-
A1	EV	MPS	100.0 %	-	B5	LH	MPS	100.0 %	-
A2	SN	MPS	100.0 %	-	B6	JV	MPS	100.0 %	-
A2	HF	R	52.22 %	-	B6	PV	MPS	100.0 %	-
A3	KO	SR	85.4 %	6.8 %	B6	KN	MPS	100.0 %	-
A4	NN	SR	61.8 %	4.0 %	B7	VF	MPS	100.0 %	-
A4	SL	SR	63.9 %	10.5 %	B7	1HV	MPS	100.0 %	-
A4	KV	SR	94.8 %	1.4 %	B7	2HV	R	28.3 %	-
A4	SH	SR	96.3 %	1.9 %	B7	HM	MPS	100.0 %	-
A4	OF	SR	95.2 %	2.4 %	B8	HO	MPS	100.0 %	-

The Ct-values obtained from salmon in Area A2-4 (apart from HF) are more diverse. The sampling (SR) was performed independent of any known PRV1 status and HSMI outbreak. This resulted in sampling of both dead/moribund fish as well as seemingly healthy individuals to meet the number of samples required. This sampling strategy do probably explain the variation in prevalence and Ct-values, as well as the number of PRV1-negative individuals. By looking at the mortalities at time of sampling most of the sites were in a viremic phase, as most of the sites had elevated mortalities. Still, not all the sampled salmons were positive of PRV1, and several individuals had a high Ct-value (> 30.0). Both the PRV1b and PRV1a clade was present in area A (Finnmark).

Table 7. Table showing the screening results using the universal PRV1 qPCR assay on the sample set collected from one semi closed containment system (SCCS) and its open reference net pen at site 2HV. The site was sampled eight times during 4-month time span after sea transfer. The number of total samples as well as number of positive samples in each sampling is included, as well as the respective Ct-values from each positive sample.

Site: 2HV						
Net pen:	**SCCS**			**O**		
	N total samples	N positive samples	Ct-value	N total samples	N positive samples	Ct-value
Sampling 0	30	0		-	0	
Sampling 1	29	1	37.1	30	1	35.1
Sampling 2	30	0		30	0	
Sampling 3	30	0		30	0	
Sampling 4	30	1	39.8	30	0	
Sampling 5	30	0		25	0	
Sampling 6	30	0		30	0	
Samplng 7	30	2	23.3, 38.0	30	3	39.1, 39.9, 39.6
Sampling 8	30	6	17.1, 35.0, 34.5, 33.6, 34.7, 38.1	30	2	21.9, 39.6

The overall prevalence of PRV1 was high. The dataset represents most active production sites for a Norwegian salmon producer, and the study shows that the sampled salmon populations had an average prevalence of 88.9 % at the sampling point. A total of 1475 salmon have been analyzed originating from 19 sites stocked in the period of 2018-2020. This provides, to the best of my knowledge, the most comprehensive study of PRV1 genotypes and HSMI on farmed salmon in Arctic Norway.

3.6 Phylogenetic analyses of PRV1 based on segments S1 and M2 from PRV1

The phylogenies presented are based 1951 and 960 nucleotides within the ORF of segments M2 and S1, respectively (Figure 8 and 9). All the S1 and M2 sequences presented in this study group within two major clades where clade PRV1b is putative high virulent and being able to cause HSMI while the clade PRV1a contains isolates believed to be less virulent and not being able to cause HSM. These two major clades can be further subdivided. PRV1a can be divided into a clade with viruses from NAPC, three clades from Norway, and one clade from the Faeroes. There are less genetic differences between the viruses in PRV1b except for one virus (OF2020/9). A novel variant identified in this study and collected from a farm in Finnmark county.

The oldest sequence isolates of PRV1 can be found in clade PRV1a (NOR-1988 and NOR-1996), while the oldest sequences in clade PRV1b are NOR-1997 and NOR-2005/TT. The first genome

obtained from PRV1 was from NOR-2010, belonging to clade PRV1b, and originated from salmon suffering from HSMI. Four of the new sequences group in clade PRV1a based on the analyses of both M2 and S1 (KO-3, KO-5, OF-10, NN-12), while EV-27 and SV-26, based on analysis of M2, and OF-x9 and SL-1, based analysis of S1, respectively, group in clade PRV1a based on one of the segments only. All the remaining new PRV1 sequences of M2 and S1 belong to clade PRV1b. The fact that the two segments from EV27, SV-26 and SL-1 belong to both clade PRV1a and PRV1b show that reassortment of the segments must have occurred. A double infection is observed in the two salmon from where the sequences OF- 9, OF-x9, OF-10 and OF-x10 were obtained. Two different RNA extraction from tissues of these two salmon, followed by sequencing of segments S1, resulted in two different S1 sequences belonging to both clade PRV1a and PRV1b.

When it comes to the qPCR from the PRV1-A3 assay (PRV1b specific assay), the results SL-1, KO-3, OF-10, and NN-12 were identified correctly into the PRV1a clade. However, some challenges were detected when using the PRV1-A3 assay to differentiate genogroup PRV1a and PRV1b. The assay did not place KO-5 in clade PRV1a. This is probably due to a double infection with isolates from both genogroups in this salmon. When looking at the analysis of the M2 segment, the sequence isolates SV-26 and EV-27 were shown to belong in the PRV1a clade, while the S1 segment places the PRV1 from these two fish in clade PRV1b. The sequence isolate SL-1 was grouping with the PRV1a group in segment S1, which the assay predicted. The sequence isolate makes a shift to PRV1b when looking at M2, which may indicate a reassortment in the isolate. Figure 10 shows that the assay only detects the members of clade PRV1b, with some exceptions on the forward primer.

Table 8 gives an overview of obtained sequences with the site and fish code, year of sampling, sequence code and what clade the sequenced isolate grouped in, based on S1 and M2. Table 9 and 10 gives an overview of differing amino acids appearing in the sequenced isolates, based on both S1 and M2, where the differing amino acids are highlighted.

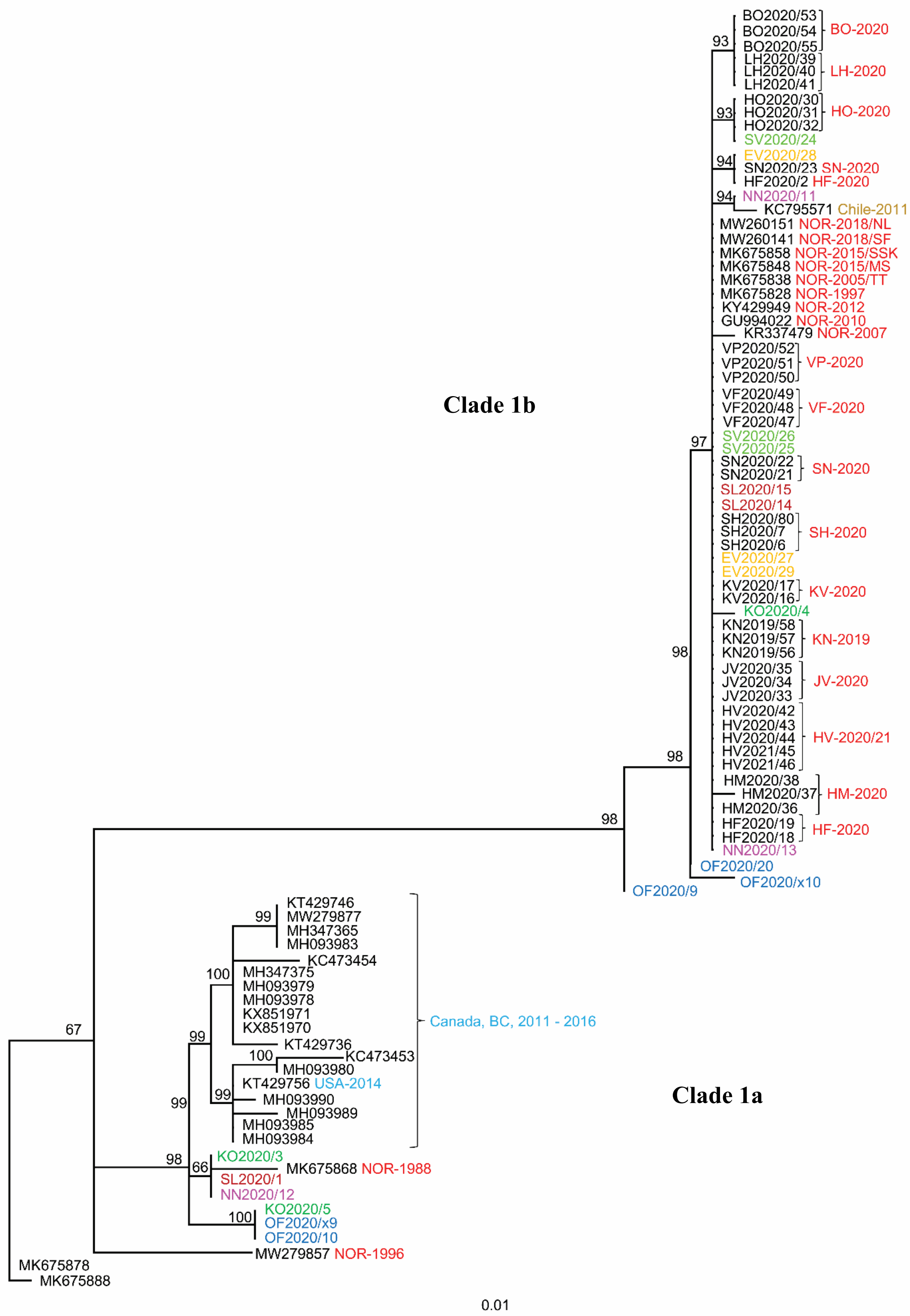


Figure 8 S1 PRV1. Phylogenetic tree showing the relationships between PRV1 sequences of segment S1 obtained from salmon included in the study. Sequences of the S1 from PRV1 obtained from Faeroe Islands (Accession no: MK675888) has been used as outgroup. The analysis is based on 960 nucleotides. The scale bar shows the number of nucleotide substitutions as a proportion of branch lengths.

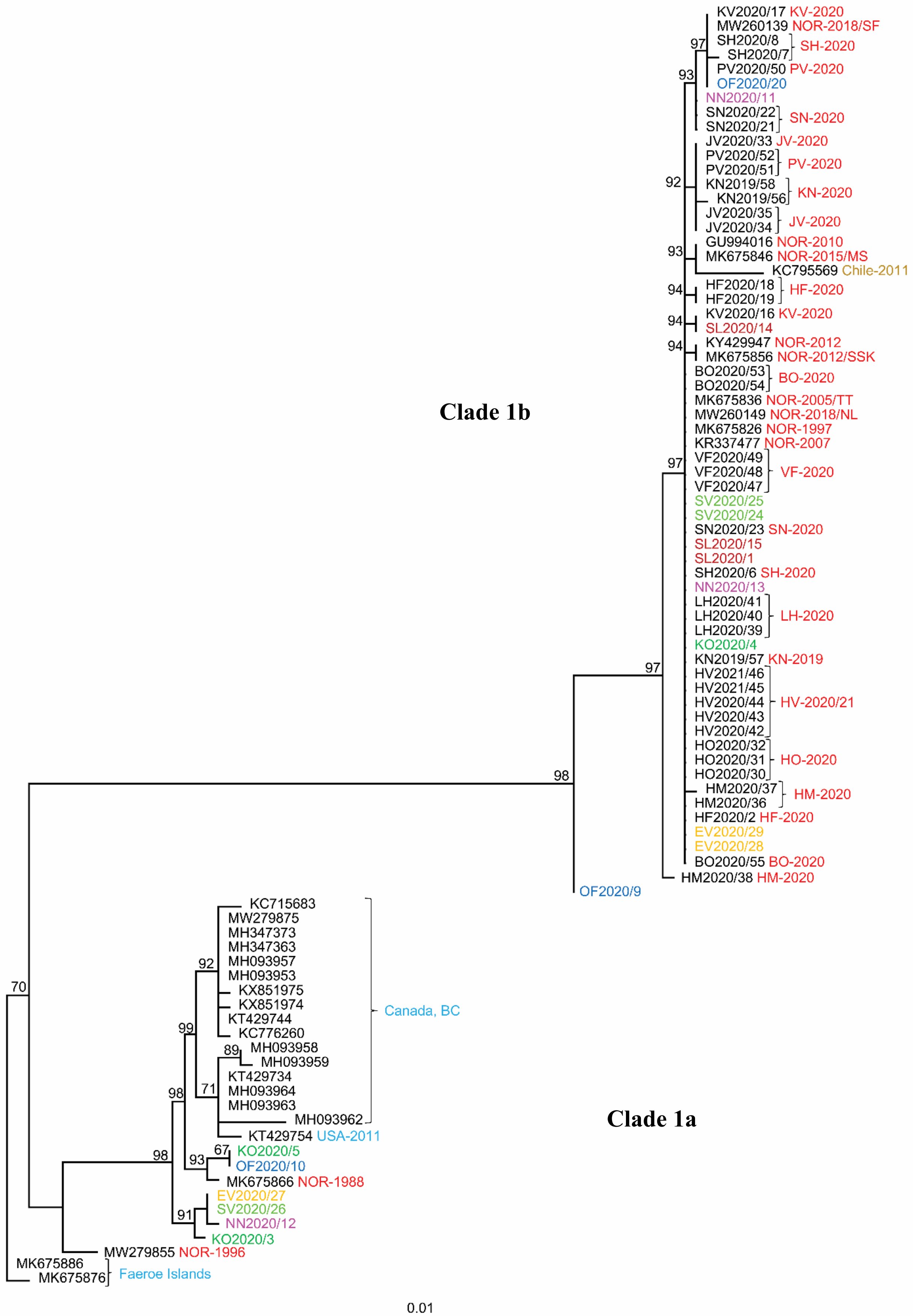


Figure 9 M2 PRV1. Phylogenetic tree showing the relationships between PRV1 sequences of segment M2 obtained from salmon included in the study. Sequences of the M2 from PRV1 obtained from Faeroe Islands (Accession no: MK675876) has been used as outgroup. The analysis is based on 1951 nucleotides. The scale bar shows the number of nucleotide substitutions as a proportion of branch lengths.

Table 8. Sequences of segments M2 and S1 obtained from salmon included in this study. The area code indicates where the site is located (Figure 1). The code gives the numbers of the individual fish. The third column shows if the site has an HSMI diagnosis or another diagnosis. The next column presents the sequence name (PRV1), and the phylogenetic position of M2 and S1 sequences with respect to the two major clades, PRV1a and PRV1b. NA = Not available.

				Clade	Clade					Clade	Clade
Area	**Fish no.**	**Diagnosis**	**PRV1**	**M2**	**S1**	**Area**	**Fish no.**	**Diagnosis**	**PRV1**	**M2**	**S1**
A1	SV-24	HSMI	SV2020-24	1b	1b	B5	BO-53	HSMI	BO2020-53	1b	1b
	SV-25	HSMI	SV2020-25	1b	1b		BO-54	HSMI	BO2020-54	1b	1b
	SV-26	HSMI	SV2020-26	**1a**	1b		BO-55	HSMI	BO2020-55	1b	1b
A1	EV-27	HSMI	EV2020-27	**1a**	1b	B5	LH-39	Other	LH2020-39	1b	1b
	EV-28	HSMI	EV2020-28	1b	1b		LH-40	Other	LH2020-40	1b	1b
	EV-29	HSMI	EV2020-29	1b	1b		LH-41	Other	LH2020-41	1b	1b
A2	SN-21	HSMI	SN2020-21	1b	1b	B6	JV-33	HSMI	JV2020-33	1b	1b
	SN-22	HSMI	SN2020-22	1b	1b		JV-34	HSMI	JV2020-34	1b	1b
	SN-23	HSMI	SN2020-23	1b	1b		JV-35	HSMI	JV2020-35	1b	1b
A2	HF-2	HSMI	HF2020-2	1b	1b	B6	PV-50	NA	PV2020-50	1b	1b
	HF-18	HSMI	HF2020-18	1b	1b		PV-51	NA	PV2020-51	1b	1b
	HF-19	HSMI	HF2020-19	1b	1b		PV-52	NA	PV2020-52	1b	1b
A3	KO-3	HSMI	KO2020-3	**1a**	**1a**	B6	KN-56	HSMI	KN2019-56	1b	1b
	KO-4	HSMI	KO2020-4	1b	1b		KN-57	HSMI	KN2019-57	1b	1b
	KO-5	HSMI	KO2020-5	**1a**	**1a**		KN-58	HSMI	KN2019-58	1b	1b
A4	NN-11	HSMI	NN2020-11	1b	1b	B7	VF-47	HSMI	VF2020-47	1b	1b
	NN-12	HSMI	NN2020-12	**1a**	**1a**		VF-48	HSMI	VF2020-48	1b	1b
	NN-13	HSMI	NN2020-13	1b	1b		VF-49	HSMI	VF2020-49	1b	1b
A4	SL-1	HSMI	SL2020-1	1b	**1a**	B7	1HV-42	Other	1HV2020-42	1b	1b
	SL-14	HSMI	SL2020-14	1b	1b		1HV-43	Other	1HV2020-43	1b	1b
	SL-15	HSMI	SL2020-15	1b	1b		1HV-44	Other	1HV2020-44	1b	1b
A4	KV16	HSMI	KV2020-16	1b	1b	B7	2HV-45	Other	1HV2020-45	1b	1b
	KV17	HSMI	KV2020-17	1b	1b		2HV-46	Other	1HV2020-46	1b	1b
A4	SH-6	HSMI	SH2020-6	1b	1b	B7	HM-36	HSMI	HM2020-36	1b	1b
	SH-7	HSMI	SH2020-7	1b	1b		HM-37	HSMI	HM2020-37	1b	1b
	SH-8	HSMI	SH2020-8	1b	1b		HM-38	HSMI	HM2020-38	1b	1b
A4	OF-9	HSMI	OF2020-9	1b	**1b-1a**	B8	HO-30	HSMI	HO2020-30	1b	1b
	OF-10	HSMI	OF2020-10	**1a**	**1b-1a**		HO-31	HSMI	HO2020-31	1b	1b
	OF-11	HSMI	OF2020-11	1b	1b		HO-32	HSMI	HO2020-32	1b	1b

Table 9. Unique amino acids (aa) in the open reading frame, σ3, from segment S1 is emphasized with a different background color.

Segment S1	**Posisjon** aa	39 P/S	69 V/T	78 D/E	85 T/A	90 T/A	117 N/T	137 V/I	156 T/A	157 A/S	164 G/D	174 E/K	206 A/V	218 V/I	226 T/A	252 H/Q
Clade PRV1a																
KO2020/3		P	T	E	A	T	T	I	A	S	G	K	V	I	T	H
KO2020/5		P	T	E	A	T	T	I	A	S	G	K	V	I	T	H
NN2020/12		P	T	E	A	T	T	I	A	S	G	K	V	I	T	H
OF2020/10		P	T	E	A	T	T	I	A	S	G	K	V	I	T	H
OF2020/x9		P	T	E	A	T	T	I	A	S	G	K	V	I	T	H
SL2020/1		P	T	E	A	T	T	I	A	S	G	K	V	I	T	H
MK675868		S	T	E	A	T	T	I	A	S	G	K	V	I	T	H
MK675878		P	T	E	A	T	T	I	A	S	G	K	V	I	T	H
MK675888		P	T	E	A	T	T	I	A	S	G	K	V	I	T	Q
MW279857		P	T	E	A	T	T	I	A	S	G	K	V	I	T	H
Clade PRV1b																
Alle		P	V	D	T	T	N	V	T	A	G	E	A	V	T	H
OF2020-20		P	V	D	A	T	N	V	T	A	G	E	A	V	T	H
HO2020/30		P	V	D	T	A	N	V	T	A	G	E	A	V	T	H
HO2020/31		P	V	D	T	A	N	V	T	A	G	E	A	V	T	H
HO2020/32		P	V	D	T	A	N	V	T	A	G	E	A	V	T	H
SV2020/24		P	V	D	T	A	N	V	T	A	G	E	A	V	T	H
KR337479		P	V	D	T	T	N	V	T	A	D	E	A	V	T	H
BO2020/53		P	V	D	T	T	N	V	T	A	G	E	A	V	A	H
BO2020/54		P	V	D	T	T	N	V	T	A	G	E	A	V	A	H
BO2020/55		P	V	D	T	T	N	V	T	A	G	E	A	V	A	H
LH2020/39		P	V	D	T	T	N	V	T	A	G	E	A	V	A	H
LH2020/40		P	V	D	T	T	N	V	T	A	G	E	A	V	A	H
LH2020/41		P	V	D	T	T	N	V	T	A	G	E	A	V	A	H
OF2020-9		P	V	D	T	T	N	V	A	S	G	E	A	V	T	H

Table 10. Unique amino acids (aa) in the open reading frame, µ1, from segment M2 is emphasized with a different background color.

Segment M2	**Posisjon** aa	68 Y/H	141 I/T	184 T/S	262 S/A	320 A/T	370 D/N	389 V/A	397 T/A	406 K/R
Clade PRV1a										
EV2020-27		Y	I	S	A	A	N	V	T	K
KO2020-3		Y	I	S	A	A	N	V	T	K
KO2020-5		Y	I	S	A	T	N	V	T	K
NN2020-12		Y	I	S	A	A	N	V	T	K
OF2020-10		Y	I	S	A	T	N	V	T	K
SV2020-26		Y	I	S	A	A	N	V	T	K
MK675866		Y	I	S	A	A	N	A	T	K
MW279855		Y	I	S	A	A	N	V	T	K
MK675876		Y	T	S	A	A	N	V	T	K
MK675886		Y	I	S	S	A	N	V	T	K
Clade PRV1b										
Alle		Y	I	T	S	A	D	V	T	K
KN2019-56		H	I	T	S	A	D	V	T	K
HM2020-37		Y	I	T	S	A	D	V	A	K
SH2020-7		Y	I	T	S	A	D	V	T	R

```
EV2020/27   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
SV2020/26   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
KO2020/5    : GTGTTGAATGTGGGCATCGGGTTGGTCTGTCTCATAACGCTAAGCCTACTATGGTCACTCATCAATGCGATGGCGAA
NN2020/12   : GTGTTGAATGCGGGCATCGGGTTGGTCTGTCTCATAACGCTAAGCCTACTATGGTCACTCATCAATGCGATGGCGAA
OF2020/10   : GTGTTGAATGTGGGCATCGGGTTGGTCTGTCTCATAACGCTAAGCCTACTATGGTCACTCATCAATGCGATGGCGAA
OF2020/x9   : GTGTTGAATGTGGGCATCGGGTTGGTCTGTCTCATAACGCTAAGCCTACTATGGTCACTCATCAATGCGATGGCGAA
SL2020/1    : GTGTTGAATGCGGGCATCGGGTTGGTCTGTCTCATAACGCTAAGCCTACTATGGTCACTCATCAATGCGATGGCGAA
MK675878    : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAACGCTAAGCCTACTATGGTCACTCATCAATGCGATGGCGAA
MK675868    : GTGTTGAATGCGGGCATCGGGTTGGTCTGTCTCATAACGCTAAGCCTACTATGGTCACTCATCAATGCGATGGCGAA
MW279877    : GTGTTGAATGCGGGCATCGGGTTGGTCTGTCTCATAACGCTAAGCCTACTATGGTCACTCATCAATGCGATGGCGAA
MK675888    : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAACGCTAAGCCTACTATGGTCACTCATCAATGCGATGGCGAA
MW279857    : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAACGCTAAGCCTACTATGGTCACTCATCAATGCGATGGCGAA
BO2020/53   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
BO2020/54   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
BO2020/55   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
EV2020/28   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
EV2020/29   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
HF2020/18   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
HF2020/19   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
HF2020/2    : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
HM2020/36   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
HM2020/37   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
HM2020/38   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
HO2020/30   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
HO2020/31   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
HO2020/32   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
HV2020/42   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
HV2020/43   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
HV2020/44   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
HV2021/45   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
HV2021/46   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
JV2020/33   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
JV2020/34   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
JV2020/35   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
KN2019/56   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
KN2019/57   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
KN2019/58   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
KO2020/4    : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
KV2020/16   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
KV2020/17   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
LH2020/39   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
LH2020/40   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
LH2020/41   : GTGTTGAATGCGGGCATCGGGTTGGTTTGTCTCATAATGCTAAGCCTGTTATGGTCACTCATCAATGCGACGGCGAT
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Figure 10 The real time assay (PRV1-A3) shows that members of clade PRV1b are targeted while the primers and probe are not matching members of clade PRV1a. The exceptions are the two sequences of S1 from the PRV1 from Faeroe Islands Accession nos: MK675878 and MK675888) and one S1 sequence from Norway collected from salmon in 1996 (MW279857). All three belong to clade PRV1a but the forward primer targeting PRV1b are matching these sequences.

4. Discussion

4.1 Virulence markers in well studied salmon viruses

Norwegian salmon farming has impacted the selection pressure exerted on salmon specific viruses by greatly increasing number of available hosts and the host density in geographical areas. It has been theorized that this has changed the virulence of previously well-adapted, salmon specific viruses. The virulence of viruses infecting farmed salmon have been subject of focus the past two decades. There are no effective vaccines available against the most challenging viral diseases in the industry, and virulent viruses can therefore cause severe diseases outbreaks resulting in high mortalities and severe economic losses in the industry. By identifying specific virulence markers, the virulence of an isolate could be predicted. Some of the most important viral salmonid diseases found in Norway are infectious salmon anemia (ISA), caused by infectious salmon anemia virus (ISAV), infectious pancreatic necrosis (IPN) caused by infectious pancreatic necrosis virus (IPNV), and heart- and skeletal muscle inflammation (HSMI) caused by *Piscine orthoreovirus* (PRV1) (Håstein & Krogsrud, 1976; Krossøy, Hordvik, Nilsen, Nylund, & Endresen, 1999; Palacios et al., 2010; Plarre, 2012; Thorud & Djupvik, 1988). The work on identifying specific virulence markers/factors has been both successful (ISAV) and unsuccessful (IPNV), where reliable makers for virulence have been identified in ISAV but not for IPNV.

ISAV is one of the most studied viruses causing mortalities in farmed Atlantic salmon. The only natural reservoir for this virus found in the North Atlantic are salmonids as *S. salar* and *S. trutta* (Plarre, 2012). Two virulent markers have been identified in ISAV. The low virulent HPR0 changed to a high virulent HPRΔ as a result of inserts or mutation in the fusion protein (F) in addition to changes in the HPR-region of the HE protein (Devold, Karlsen, & Nylund, 2006; Markussen et al., 2008; McBeath, Bain, & Snow, 2009; A. Nylund, Devold, Plarre, Isdal, & Aarseth, 2003; A. Nylund et al., 2007; Plarre, 2012). The HPR0 and HPRΔ ISAV have a clear difference in virulence and the identification of the virulence markers have been a motivation for finding similar virulence markers in other fish viruses present in Norwegian aquaculture.

IPN is another severe salmonid disease causing mortality among salmon, rainbow trout fry and postsmolts (Roberts & Pearson, 2005). The losses caused by this disease frequently reaches 80 – 90 % of the affected stocks. This has resulted in great efforts of finding ways of controlling

the disease, and studies attempting to identify virulence markers have been carried out (Santi, Song, Vakharia, & Evensen, 2005; Song, Santi, Evensen, & Vakharia, 2005). The virulence markers suggested in these studies have been questioned (Smail et al., 2006) and later refuted (Dopazo, 2020). Hence, there are no well-defined virulence markers for IPNV that can be used to predict the level of virulence among isolates based on their molecular characteristics.

Observations of PRV1 positive populations lacking the occurrence of HSMI in Canada-BC, and the difference of severity of HSMI in salmon farms in Norway and Chile has led to a search for possible virulence markers in the genome of this virus. PRV1 have been describes as ubiquitous in Norwegian farmed salmon. However, the outcome of the infection has been difficult to predict. Studies of the phylogenetic relationships between PRV1 "isolates" have shown that, based on segments M2 and S1, the virus sequence isolates group in two distinct clades, PRV1a and PRV1b (Garseth, Fritsvold, Opheim, Skjerve, & Biering, 2012; Kibenge et al., 2013). Based on the results from field outbreaks of HSMI and challenge experiments it has been claimed that these two clades represent members of PRV1 with different virulence (Dhamotharan et al., 2019). Later studies have suggested that additional segments (L1, L2 and S4) had to be included separate between PRV1 "isolates" with respect to pathogenicity (Wessel, Hansen, Dahle, et al., 2020).

The aim of the present study was to map the genotypes of PRV1 associated with outbreaks of HSMI in selected farms in Finnmark and Nordland counties. However, the results show that PRV1b is present in all farms included in the study, even in the farms (LH, PV, 1HV and 2HV) that did not get a HSMI diagnosis. This shows that the PRV1b clade is the dominating genogroup infecting farmed salmon in Norther Norway, while none of the sampled populations were positive for PRV1a only. Therefore, it was not possible to further elucidate the putative low virulence and no HSMI status of PRV1a. Members of both clades co-occur in populations at some production sites. In these cases, the PRV1a was found at a lesser frequency in populations compared to PRV1b. Although the study has a suboptimal sampling regime, the number of salmon and sites included in the study strongly indicate that PRV1b is the dominating variant in farmed salmon in Northern Norway. In this study, members of both clades were present at six sites in Area A (Finnmark), while only members of clade PRV1b were present in the sites in Area B (Nordland). Based on the data from the present study it is not possible to confirm if the segments M2 and S1 can be used as virulence markers. Further work on this

material should include additional sequencing of the segments L1, L2 and S4 to see if the hypothesis presented by Wessel et al. (2020) could be supported or refuted.

4.2 Prevalence of PRV-1 in Atlantic salmon farmed in Arctic Norway

The prevalence of PRV1 in salmon at marine production sites along the Norwegian coast is believed to be high (Polinski et al., 2020). When looking at Table 6, the prevalence is overall high in both Areas included in this study. In Area A1 and B5-8 the samples was chosen based on confirmed PRV1 positive samples with a low Ct-value. The low Ct-values could indicate that the virus was in a viremic phase, and salmon at most of these sites (SV, EV, BO, JV, KN, HM, HO) developed HSMI. The sites in Area A2-4 had a varying prevalence of PRV1 with elevated mortalities at sampling point, which may indicate that the virus was in a viremic phase in these sites as well.

Three of the sites in the study (LH, 1HV, 2HV) did not get an official HSMI diagnosis during the study period. It should be added that the official diagnosis for the salmon kept at site PV has not been made available for this study. No CF mortality category was available for LH or 1HV. The salmon farmed at site 2HV were followed for 110 days (15th of September until 4th of January) only, and it cannot be excluded that this population may also develop HSMI later in production.

These data support previous studies showing that PRV1 and HSMI is common in the production of farmed salmon (Løvoll et al., 2012; Sommerset et al., 2021). The fact that PRV1 has been allowed to spread among farmed salmon through movements of smolt and horizontal transmission between farms could have resulted in reduced prevalence of the low-virulent type and increased prevalence of the virulent type. However, none of these PRV1 "isolates" belonging to PRV1a or PRV1b result in mortalities in challenge experiments (Di Cicco et al., 2017; Wessel et al., 2017) suggesting that other factors may be needed for the observed mortality in marine production of salmon.

The sequence information obtained from segments M2 and S1 from PRV1 "isolates" included in this study show that the majority belong in clade PRV1a or PRV1b as seen in other studies from Norway (Dhamotharan et al., 2019, Wessel et al. 2020). However, in the present study it is shown for the first time reassortment between members of clade PRV1a and PRV1b. PRV1 "isolates" SV2020-26 and EV2020-27, both from the same area (A1) in Finnmark, contain a

M2 segment belonging to PRV1a and an S1 segment from PRV1b. The close distance between these two sites could indicate that this strain of PRV1 has been spread horizontally between the sites since the origin of the smolt delivered to the two sites were different. This area, if to be used for salmon production should be monitored in the coming years to see if this strain may establish in this area or if it will be outcompeted by the members of PRV1b that seem to be dominating in A1. It is to be expected that the most virulent strain should dominate and if members of PRV1b are representing the most virulent strains the PRV1-M2a-S1b should be lost. The same should be expected for the other PRV1 reassortant (SL2020-1: PRV1-M2b-S1a) in area A4. Double infections with both PRV1a and PRV1b were also observed in two salmon in area A4 where PRV1 "isolates" present in salmon OF-9 and OF-10 contain segment S1 from both clade PRV1a and PRV1b. The former had a M2 segment belonging to clade PRV1b while the latter had a M2 segment belonging to clade PRV1a. This was discovered after two separate RNA extractions followed by sequencing of both segments M2 and S1 was performed. This is allegedly, the first observation of a doble infection in the same individual salmon.

4.2.1 Specificity of the PRV1-A3 qPCR assay

An assay designed to target the putative high virulent variant, PRV1b, was tested during this study. This is the first big scale testing conducted with this assay. The assay (PRV1-A3) is designed by Siah et al. (2020) and specifically targets the S1 segment. The Ct-values obtained with this assay was on average 1-2 numbers lower than the ones obtained with the M2-assay. Because of the increased specificity towards the PRV1b variant this decline is expected. When analyzing confirmed PRV1-positive samples some of the samples was "Negative" (Neg) and presumably a putative low virulent variant, PRV1a. A selection of these samples was sequenced, and phylogenetic analyses were conducted to either confirm or refute the obtained qPCR results.

The sequenced isolates classified as low virulent using the assay was SL-1, KO-3, OF-9, and NN-12. The phylogenetic analyzes at S1 (Figure 8) confirms the clade of these isolates. Surprisingly, KO-5 also group in this clade. This isolate was positive when using the PRV1-A3 assay (Ct = 25.2). The Ct-value of the PRV1 universal M2-screening was lower, at 19.5, which differ from the other Ct-values which typically declined when using PRV1-A3. The likely explanation for this result is a co-infection in the salmon resulting in different sequences and qPCR result. KO-3 and NN-12 are still grouping with the low virulent variants when looking at M2 (Figure 9). Sl-1 and OF-9, considered as low virulent by the assay, convert to the high

virulent clade. KO-5 is still grouping with the low virulent group. The assumed high virulent isolates OF-10, SV-26 and EV-27 all group with the low virulent group when looking at M2.

4.3 The geographical distribution of the obtained isolates

There is little variation in the isolates obtained, both at sites in areas geographical close to each and in between areas with a significant geographic distance. Horizontal transmission of PRV1 have been shown, and is believed to be associated with ocean currents as well as between sites sharing personnel and equipment (Uglem, Dempster, Bjørn, Sanchez-Jerez, & Økland, 2009). The risk of a new infection of PRV1 was significantly increased on farms with previously infected populations (Aldrin et al., 2009). This suggest the maintenance of local reservoirs in other farmed salmon or in wild salmon, where farmed salmon are believed to be of most important for the spreading of PRV1 (Garseth, Ekrem, & Biering, 2013). It has not yet shown experimentally that PRV1 can be vertically transmitted, but the virus may contaminate the reproductive products of PRV1 positive broodfish (Løvoll et al., 2012). In addition, contemporary PRV1 "isolates" from both Chile and Canada-BC show a close identity to PRV1 viruses from Norway and, since Atlantic salmon do not naturally occur in Chile or Pacific Canada, it is reasonable to suggest that PRV1 have been introduced to these two areas via movement of salmon embryos (Kibenge et al., 2013).

PRV1 have also been detected in freshwater facilities in Norway meaning that movement of smolt could play an important role in the transmission of the virus in Norwegian salmon framing. The presence of PRV1 in smolt could be a result of vertical transmission or introduction via water intake to the smolt production sites. PRV1 is a naked virus that is resistant to adverse physical conditions and disinfection. Without proper disinfection of the equipment such as tanks and pipelines, the risk of transmission to the next generation is increased. Most of the obtained isolates are identical with few genetic differences regardless of which facility the isolate originates from. This is even more prominent in isolates that differs from the majority, as the isolates from B6 (HO) grouping together with one isolate from A1 (SV) (Figures 1 and 8). Both sites share the same broodfish as well as the same freshwater nursery. One of these factors could explain the presence of identical PRV1 "isolates" at the two sites.

When looking at the amino acids of the isolates sequenced in this study there is little variations in both segments, and the variation observed reflects the genetic variation obtained from the

phylogenetic analyses. The genetic variation between the obtained isolates is larger when looking at the S1 gene, as seen by others (Dhamotharan et al., 2019; Wessel, Hansen, Dahle, et al., 2020). However, based on the present study it is not possible to claim that these unique amino acids found in PRV1a S1 or M2 represent "isolates" of lower virulence compared to members of PRV1b.

5. Conclusion and future research

The prevalence of HSMI and genetic variations of PRV1 in salmon farmed in Arctic Norway was investigated in this study. This study concludes with an overall high prevalence of PRV1 and HSMI in the investigated areas. The genetic variations of PRV1 were more significant in Area A, where both an atypical isolate as well as the 1a group was present.

HSMI was present at all but one site in this study. The population at this site was positive for a PRV1b infection. The severity of HSMI mortality observed in this study is not as severe as is indicated by literature. When looking at 19 sites, the site with the highest HSMI mortality was 3.6 %, where the most affected net pen had a mortality at 9.0 % at the end of the production cycle. The average mortality caused by HSMI in this study was at 1.0 %. By only looking at the genotype present at the site you cannot predict the severity of a HSMI outbreak.

Future research will consist of compiling be more of the same data over several generations to get an overview of how the distribution of the virus and the disease is evolving over time. By comparing data from several generations at the same site, a pattern as to what may trigger the disease can occur. The sampling must be done as similarly as possible to ensure a more probable prevalence and distribution of the different subgenotypes present in the area.

The virus isolates sequenced in this study could be sequenced at the other segments as well, to increase the resolution on strain level. The novel isolate variant should be whole genome sequenced to best support current knowledge on genetic variation in PRV1.

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Appendix

Recipes

<u>50X TAE-Buffer (Tris-Acetate-EDTA-buffer):</u>

- Tris Base (Merck) 242 g
- Glacial acetetic acid 57.1 mL
- 0.5M EDTA (pH=8.0) 100 mL
- Add H_2O to a final volume of 1000 mL

<u>1X TAE-Buffer:</u>

- 200 mL 50X TAE-Buffer
- 10 L H_2O

<u>1,5% Agarose gel:</u>

- SeaKem® LA Agarose (Cambrex) solved in 400 mL 1X TAE-Buffer
- Heat in microwave oven and store at 60°C.

Sampling information 2HV

Table 11. Table showing details about the sampling performed at site HV 20G. The sampling was executed over 4 months. A pre stocking sampling was performed at the freshwater facility prior to sea transfer. 2 net pens were sampled, one SCCS and one open net pen (O). Both the date of sampling and number of fish sampled is included.

Site: HV 20G						
Sea transfer: 15.09.20	Sampling FW:		Sampling SW:			
Pre stocking sampling	10.09.20	30	SCCS		O	
Sampling 1			16.09.20	29	16.09.20	30
Sampling 2			23.09.20	30	23.09.20	30
Sampling 3			30.09.20	30	30.09.20	30
Sampling 4			09.10.20	30	09.10.20	30
Sampling 5			14.10.20	30	14.10.20	25
Sampling 6			09.11.20	30	10.11.20	30
Sampling 7			07.12.20	30	07.12.20	30
Sampling 8			04.01.21	30	04.01.21	30

Ct-values from all screened samples in this study

Table 12. Ct-values obtained from the site SV and the date of sampling.

Site: SV	Date: November 20	
Fish	PRV1M2	PRV1-A3
F1	16.2	13.2
F2	19.3	16.8
F3	20.5	16.3
F4	23.8	21.8
F5	22.5	18.4
F6	25.1	18.2
F7	17	13.8
F8	18	15.4

Table 13. Ct-values obtained from the site EV and the date of sampling.

Site: EV	Date: November 20	
Fish	PRV1M2	PRV1-A3
F1	21.1	18.2
F2	19.4	15.3
F3	17.7	13.0
F4	15.1	13.2
F5	19.6	14.8
F6	18.5	15.4

Table 14. Ct-values obtained from the site SN and the date of sampling.

Site: SN	Date: January 21	
Fish	PRV1M2	PRV1-A3
F1	17.2	12.9
F2	20.1	16.0
F3	20.8	17.8
F4	20.8	15.8
F5	20.6	16.8
F6	25.9	18.1
F7	20.6	18.7
F8	20.6	16.4
F9	18.3	14.5
F10	24.4	16.9

Table 15. Ct-values obtained from the site HF and the date of sampling.

Site: HF				Date: September/November 20	
Fish	PRV1M2	Fish	PRV1M2	Fish	PRV1M2
F1	Neg	F41	Neg	F81	Neg
F2	Neg	F42	22.9	F82	Neg
F3	Neg	F43	Neg	F83	36.0
F4	Neg	F44	35.0	F84	Neg
F5	Neg	F45	35.7	F85	31.8
F6	Neg	F46	23.7	F86	Neg
F7	Neg	F47	Neg	F87	Neg
F8	Neg	F48	25.2	F88	30.2
F9	Neg	F49	31.1	F89	Neg
F10	Neg	F50	33.8	F90	Neg
F11	Neg	F51	38.7	F91	Neg
F12	Neg	F52	23.4	F92	35.0
F13	Neg	F53	33.8	F93	31.8
F14	Neg	F54	Neg	F94	Neg
F15	Neg	F55	Neg	F95	Neg
F16	Neg	F56	Neg	F96	Neg
F17	Neg	F57	18.8	F97	Neg
F18	Neg	F58	22.5	F98	Neg
F19	Neg	F59	26.8	F99	35.2
F20	Neg	F60	21.1	F100	Neg
F21	Neg	F61	Neg	F101	36.1
F22	Neg	F62	Neg	F102	17.7
F23	20.9	F63	Neg	F103	Neg
F24	Neg	F64	20.8	F104	36.4
F25	Neg	F65	36.6	F105	36.9
F26	Neg	F66	Neg	F106	33.3
F27	Neg	F67	34.7	F107	Neg
F28	35.2	F68	35.1	F108	36.6
F29	36.0	F69	Neg	F109	Neg
F30	Neg	F70	Neg	F110	34.1
F31	Neg	F71	Neg	F111	37.3
F32	34.2	F72	36.5	F112	Neg
F33	Neg	F73	35.9	F113	Neg
F34	Neg	F74	Neg	F114	Neg
F35	35.9	F75	Neg	F115	Neg
F36	35.5	F76	Neg	F116	Neg
F37	35.3	F77	Neg	F117	Neg
F38	33.2	F78	Neg	F118	Neg
F39	Neg	F79	Neg	F119	Neg
F40	33.7	F80	36.1	F120	Neg

Table 16. Ct-values obtained from the site KO and the date of sampling. Samples believed to be putative low virulent (PRV1a) based on the assay are emphasized.

Site: KO			Date: July/August 20		
Fish	PRV1M2	PRV1-A3	Fish	PRV1M2	PRV1-A3
F1	34.8	31.3	F21	25.0	22.4
F2	33.6	30.3	F22	Neg	Neg
F3	19.9	Neg	F23	36.0	33.5
F4	32.0	31.7	F24	33.2	31.2
F5	34.9	33.8	F25	35.9	36.5
F6	34.4	32.1	F26	30.0	27.2
F7	37.3	Neg	F27	33.7	31.0
F8	Neg	Neg	F28	30.3	28.6
F9	37.2	Neg	F29	35.2	34.6
F10	35.6	33.3	F30	29.2	24.9
F11	32.6	31.5	F31	33.4	32.6
F12	36.2	32.0	F32	34.7	32.3
F13	19.5	25.2	F33	20.8	19.3
F14	23.8	21.6	F34	26.8	24.3
F15	21.3	19.2	F35	32.1	29.5
F16	28.9	26.7	F36	33.9	30.6
F17	28.8	27.0	F37	30.8	28.4
F18	23.2	20.6	F38	20.7	18.3
F19	32.4	30.9	F39	22.7	20.4
F20	33.9	32.5	F40	25.5	22.6

Table 17. Ct-values obtained from the site NN and the date of sampling. Samples believed to be putative low virulent (PRV1a) based on the assay are emphasized.

Site: NN				Date: July 20				
Fish	PRV1M2	PRV1-A3	Fish	PRV1M2	PRV1-A3	Fish	PRV1M2	PRV1-A3
F1	22.4	20.6	F88	37.4	32.8	F175	30.1	28.4
F2	21.6	19.3	F89	Neg	Neg	F176	30.9	29.1
F3	Neg	Neg	F90	Neg	Neg	F177	20.3	18.6
F4	Neg	Neg	F91	28.5	25.2	F178	20.5	19.0
F5	Neg	Neg	F92	Neg	Neg	F179	20.6	19.4
F6	Neg	Neg	F93	Neg	Neg	F180	37.2	36.3
F7	Neg	Neg	F94	22.8	18.7	F181	28.6	26.5
F8	Neg	Neg	F95	Neg	Neg	F182	19.1	17.0
F9	Neg	Neg	F96	35.3	30.4	F183	29.4	27.7
F10	Neg	Neg	F97	21.5	18.8	F184	36.0	36.2
F11	Neg	Neg	F98	36.5	32.7	F185	29.8	28.2
F12	Neg	Neg	F99	Neg	Neg	F186	22.1	20.5
F13	29.4	27.2	F100	34.9	32.8	F187	21.1	19.6
F14	Neg	Neg	F101	21.5	17.6	F188	34.5	30.8
F15	27.0	25.0	F102	33.4	30.5	F189	29.9	28.7
F16	Neg	Neg	F103	36.5	34.2	F190	33.6	32.7
F17	Neg	Neg	F104	27.9	22.8	F191	27.8	26.9
F18	Neg	Neg	F105	34.2	29.8	F192	Neg	Neg
F19	Neg	Neg	F106	34.0	30.7	F193	35.6	33.8
F20	Neg	Neg	F107	23.2	19.3	F194	19.4	17.6
F21	Neg	Neg	F108	Neg	Neg	F195	33.9	33.3
F22	Neg	Neg	F109	Neg	Neg	F196	36.8	39.5
F23	Neg	Neg	F110	25.6	21.4	F197	26.7	24.9
F24	Neg	Neg	F111	25.6	22.6	F198	26.9	36.0
F25	Neg	Neg	F112	Neg	Neg	F199	25.3	23.6
F26	Neg	Neg	F113	21.5	19.6	F200	27.2	25.3
F27	Neg	Neg	F114	32.1	32.9	F201	Neg	Neg
F28	Neg	Neg	F115	24.8	Neg	F202	Neg	Neg
F29	Neg	Neg	F116	32.5	28.7	F203	25.7	23.0
F30	Neg	Neg	F117	21.9	18.1	F204	Neg	Neg
F31	Neg	Neg	F118	25.5	28.4	F205	22.8	21.4
F32	Neg	Neg	F119	25.9	24.3	F206	30.8	28.0
F33	Neg	Neg	F120	23.1	20.5	F207	24.9	23.2
F34	Neg	Neg	F121	25.2	Neg	F208	29.5	27.7
F35	Neg	Neg	F122	17.9	Neg	F209	26.8	25.3
F36	Neg	Neg	F123	19.1	27.9	F210	24.9	23.2
F37	30.3	28.6	F124	22.6	Neg	F211	22.2	21.1
F38	Neg	Neg	F125	18.4	16.4	F212	21.8	20.2
F39	Neg	Neg	F126	19.9	Neg	F213	24.8	24.5
F40	Neg	Neg	F127	22.1	20.5	F214	31.5	30.0
F41	Neg	Neg	F128	29.4	26.6	F215	19.0	17.8
F42	Neg	Neg	F129	25.7	24.8	F216	30.1	26.8
F43	26.0	23.6	F130	17.9	15.3	F217	19.1	17.4

F44	Neg	Neg	F131	23.8	23.7	F218	28.0	26.5
F45	Neg	Neg	F132	19.9	21.7	F219	21.2	20.7
F46	Neg	Neg	F133	33.3	35.6	F220	25.6	23.9
F47	36.2	32.8	F134	21.6	19.8	F221	19.0	17.9
F48	Neg	Neg	F135	25.8	23.6	F222	30.8	29.3
F49	Neg	Neg	F136	35.3	35.8	F223	20.9	19.1
F50	26.5	23.9	F137	26.5	23.6	F224	20.8	19.7
F51	Neg	Neg	F138	25.0	Neg	F225	28.9	27.1
F52	Neg	Neg	F139	26.5	27.0	F226	27.7	25.2
F53	Neg	Neg	F140	32.6	32.2	F227	29.4	27.2
F54	28.7	23.8	F141	Neg	Neg	F228	33.9	32.9
F55	Neg	Neg	F142	Neg	Neg	F229	19.4	17.6
F56	24.8	19.4	F143	Neg	Neg	F230	26.2	25.6
F57	30.1	25.7	F144	Neg	Neg	F231	28.2	27.2
F58	Neg	Neg	F145	Neg	Neg	F232	Neg	Neg
F59	32.4	27.2	F146	22.0	20.9	F233	Neg	Neg
F60	21.9	15.6	F147	Neg	Neg	F234	Neg	Neg
F61	28.7	27.5	F148	Neg	Neg	F235	33.8	31.7
F62	22.3	16.7	F149	31.3	30.1	F236	36.0	33.9
F63	Neg	Neg	F150	16.7	14.7	F237	24.1	21.9
F64	28.0	23.6	F151	21.5	19.8	F238	21.7	20.0
F65	35.4	32.0	F152	34.0	32.3	F239	32.6	31.2
F66	Neg	Neg	F153	Neg	Neg	F240	Neg	Neg
F67	Neg	Neg	F154	Neg	Neg	F241	29.9	27.6
F68	32.6	30.0	F155	38.0	36.8	F242	Neg	Neg
F69	Neg	34.9	F156	24.4	22.6	F243	Neg	Neg
F70	Neg	Neg	F157	34.4	33.0	F244	Neg	Neg
F71	35.5	30.2	F158	22.6	21.1	F245	31.4	29.3
F72	34.5	30.5	F159	Neg	Neg	F246	27.7	25.9
F73	28.1	25.0	F160	25.6	24.0	F247	24.0	22.3
F74	28.7	24.4	F161	34.2	33.0	F248	24.3	22.7
F75	23.6	18.3	F162	34.9	32.9	F249	24.2	22.6
F76	26.1	21.6	F163	Neg	Neg	F250	22.7	20.5
F77	21.7	17.3	F164	Neg	Neg	F251	23.5	20.7
F78	Neg	Neg	F165	28.5	31.1	F252	22.5	20.9
F79	42.4	29.7	F166	25.6	23.9	F253	31.1	28.9
F80	Neg	Neg	F167	30.1	28.8	F254	Neg	Neg
F81	Neg	Neg	F168	18.8	16.8	F255	30.3	28.1
F82	Neg	Neg	F169	20.5	Neg	F256	19.9	18.1
F83	23.0	18.4	F170	33.1	34.7	F257	32.4	30.3
F84	36.5	32.1	F171	36.9	37.9	F258	28.3	26.0
F85	Neg	Neg	F172	34.7	34.4	F259	24.9	23.3
F86	Neg	Neg	F173	21.3	19.5	F260	36.1	35.2
F87	35.9	30.7	F174	33.5	31.3	F261	21.5	19.5

Table 18. Ct-values obtained from the site SL and the date of sampling. Samples believed to be putative low virulent (PRV1a) based on the assay are emphasized.

Site: SL				Date: July 20				
Fish	PRV1M2	PRV1-A3	Fish	PRV1M2	PRV1-A3	Fish	PRV1M2	PRV1-A3
F1	37.4	34.9	F41	Neg	Neg	F81	36.8	39.1
F2	Neg	Neg	F42	Neg	Neg	F82	37.7	34.0
F3	34.5	33.1	F43	Neg	Neg	F83	30.5	Neg
F4	Neg	Neg	F44	Neg	Neg	F84	31.3	Neg
F5	Neg	Neg	F45	33.0	32.3	F85	28.4	30.7
F6	Neg	Neg	F46	40.7	33.8	F86	29.9	33.1
F7	Neg	Neg	F47	35.3	34.5	F87	33.5	33.9
F8	Neg	Neg	F48	34.9	33.1	F88	22.4	22.5
F9	36.8	35.4	F49	Neg	Neg	F89	25.6	36.9
F10	Neg	Neg	F50	36.8	35.2	F90	32.8	Neg
F11	36.2	33.6	F51	30.2	36.3	F91	34.9	Neg
F12	Neg	Neg	F52	31.8	30.7	F92	18.7	Neg
F13	Neg	Neg	F53	Neg	Neg	F93	35.7	Neg
F14	35.6	36.6	F54	Neg	Neg	F94	36.6	38.7
F15	Neg	Neg	F55	35.3	27.4	F95	30.5	Neg
F16	Neg	Neg	F56	34.3	32.4	F96	Neg	Neg
F17	Neg	Neg	F57	22.4	20.2	F97	Neg	Neg
F18	33.8	Neg	F58	20.5	18.5	F98	Neg	Neg
F19	Neg	Neg	F59	31.6	29.6	F99	Neg	Neg
F20	Neg	Neg	F60	36.0	33.7	F100	34.0	32.7
F21	Neg	Neg	F61	36.9	35.2	F101	Neg	Neg
F22	Neg	Neg	F62	25.1	23.0	F102	Neg	Neg
F23	Neg	Neg	F63	27.3	25.6	F103	32.2	29.8
F24	36.8	34.6	F64	30.6	29.5	F104	Neg	Neg
F25	36.7	Neg	F65	29.2	27.2	F105	Neg	Neg
F26	21.4	18.8	F66	34.8	33.9	F106	27.9	27.2
F27	34.5	32.5	F67	34.4	33.6	F107	34.9	34.8
F28	Neg	Neg	F68	Neg	Neg	F108	35.9	34.5
F29	Neg	Neg	F69	35.1	32.1	F109	33.4	31.3
F30	Neg	Neg	F70	35.8	36.6	F110	24.3	22.2
F31	24.8	22.4	F71	34.1	32.5	F111	26.5	24.4
F32	24.3	22.0	F72	31.6	30.0	F112	36.7	34.0
F33	29.5	27.2	F73	36.7	34.5	F113	30.9	28.2
F34	33.0	30.6	F74	22.5	21.8	F114	Neg	Neg
F35	25.1	22.6	F75	32.7	32.0	F115	22.2	21.4
F36	31.3	28.7	F76	32.3	31.4	F116	Neg	Neg
F37	Neg	Neg	F77	Neg	Neg	F117	23.3	22.0
F38	Neg	Neg	F78	Neg	Neg	F118	33.1	31.5
F39	Neg	Neg	F79	33.7	33.2	F119	32.1	30.3
F40	36.7	35.2	F80	36.8	39.1			

Table 19. Ct-values obtained from the site KV and the date of sampling. Samples believed to be putative low virulent (PRV1a) based on the assay are emphasized.

Site: KV			July 20		
Fish	PRV1M2	PRV1-A3	Fish	PRV1M2	PRV1-A3
F1	22.4	20.5	F41	35.7	35.9
F2	23.5	21.8	F42	36.5	34.6
F3	23.5	21.5	F43	Neg	36.0
F4	23.9	22.1	F44	31.8	30.4
F5	23.7	21.9	F45	32.1	30.9
F6	28.4	26.4	F46	34.9	32.9
F7	28.1	26.8	F47	29.7	28.8
F8	25.7	23.7	F48	29.2	27.8
F9	24.4	22.8	F49	30.3	28.4
F10	22.4	20.8	F50	32.2	31.0
F11	20.8	19.3	F51	20.5	18.9
F12	26.7	25.0	F52	31.0	29.4
F13	28.1	26.6	F53	23.0	21.5
F14	22.1	20.5	F54	32.8	31.4
F15	29.2	27.5	F55	26.5	25.3
F16	21.3	19.7	F56	25.6	23.5
F17	16.5	15.0	F57	30.9	29.6
F18	25.9	24.2	F58	35.6	33.3
F19	37.0	33.1	F59	33.9	33.3
F20	29.1	27.3	F60	33.7	32.0
F21	26.8	25.3	F61	35.7	32.6
F22	32.3	30.7	F62	34.0	32.1
F23	37.1	34.4	F63	35.1	33.7
F24	22.9	21.5	F64	33.2	32.4
F25	26.2	24.8	F65	35.0	34.3
F26	33.0	31.5	F66	35.4	34.7
F27	Neg	Neg	F67	34.2	33.0
F28	32.8	31.2	F68	35.0	33.2
F29	31.3	29.9	F69	31.9	30.4
F30	32.7	31.0	F70	34.3	31.8
F31	33.3	31.7	F71	34.5	33.1
F32	30.2	28.6	F72	34.8	35.0
F33	31.2	29.7	F73	32.5	32.1
F34	37.3	Neg	F74	34.2	32.6
F35	36.2	35.9	F75	34.7	34.2
F36	Neg	Neg	F76	33.7	33.2
F37	36.0	35.7	F77	35.5	35.0
F38	34.9	33.9			
F39	37.2	35.4			
F40	35.0	32.9			

Table 20. Ct-values obtained from the site SH and the date of sampling. Samples believed to be putative low virulent (PRV1a) based on the assay are emphasized.

Site: SH				Date: August 20				
Fish	PRV1M2	PRV1-A3	Fish	PRV1M2	PRV1-A3	Fish	PRV1M2	PRV1-A3
F1	32.0	28.8	F38	22.1	19.6	F75	23.2	20.2
F2	33.2	30.4	F39	33.5	29.9	F76	32.7	30.1
F3	33.0	30.8	F40	29.5	26.7	F77	22.1	18.9
F4	36.3	33.1	F41	33.0	29.4	F78	27.4	34.3
F5	32.2	29.1	F42	34.2	30.9	F79	31.9	30.7
F6	34.0	30.8	F43	33.3	29.8	F80	25.8	22.0
F7	33.3	29.6	F44	32.7	28.8	F81	32.6	30.3
F8	31.8	29.1	F45	34.4	30.6	F82	23.4	20.1
F9	32.7	30.5	F46	37.1	33.8	F83	22.8	20.1
F10	31.7	29.0	F47	33.3	30.8	F84	33.9	30.8
F11	31.8	28.5	F48	32.8	28.8	F85	31.1	29.4
F12	33.2	31.5	F49	34.2	30.2	F86	27.0	22.5
F13	26.3	23.7	F50	33.4	29.5	F87	32.9	30.2
F14	24.1	21.6	F51	26.0	22.9	F88	33.5	33.1
F15	30.1	28.0	F52	31.6	29.2	F89	31.8	28.9
F16	21.8	17.9	F53	28.3	26.1	F90	33.3	28.9
F17	31.8	28.8	F54	30.8	27.8	F91	34.1	32.8
F18	30.9	36.2	F55	27.0	23.5	F92	25.7	24.7
F19	35.5	36.2	F56	31.8	29.3	F93	30.3	28.8
F20	Neg	Neg	F57	32.0	30.0	F94	24.6	23.4
F21	36.8	35.0	F58	33.7	30.6	F95	31.3	29.9
F22	34.3	Neg	F59	34.2	31.7	F96	31.8	30.7
F23	29.9	27.1	F60	31.7	28.6	F97	32.9	31.6
F24	31.0	28.7	F61	30.6	29.9	F98	32.3	30.9
F25	32.1	32.3	F62	29.1	28.3	F99	30.2	30.2
F26	26.7	24.7	F63	25.9	24.7	F100	31.7	31.2
F27	32.1	32.8	F64	28.5	25.5	F101	31.6	30.4
F28	27.2	25.3	F65	24.3	22.9	F102	32.4	31.2
F29	23.6	33.7	F66	29.4	27.3	F103	30.1	28.2
F30	32.5	30.5	F67	26.9	23.6	F104	24.3	22.8
F31	Neg	Neg	F68	24.4	21.6	F105	25.7	24.0
F32	32.4	29.6	F69	30.0	27.4	F106	30.4	29.2
F33	36.3	35.0	F70	25.5	30.9	F107	30.6	29.6
F34	33.0	Neg	F71	24.5	21.8	F108	30.3	29.0
F35	32.1	30.4	F72	25.2	21.5	F109	33.2	33.0
F36	31.9	28.9	F73	21.4	18.7	F110	33.4	32.2
F37	22.6	19.7	F74	30.9	30.2			

Table 21. Ct-values obtained from the site OF and the date of sampling. Samples believed to be putative low virulent (PRV1a) based on the assay are emphasized. Because of the high Ct-values obtained by using the universal PRV1-M2 assay it was decided to only use the PRV1-A3 on a few, selected samples.

Site: OF				Date: August 20				
Fish	PRV1M2	PRV1-A3	Fish	PRV1M2	PRV1-A3	Fish	PRV1M2	PRV1-A3
F1	32.0		F30	21.2	Neg	F59	31.2	
F2	32.7		F31	30.5		F60	34.0	
F3	31.3		F32	26.3		F61	33.4	
F4	32.2		F33	31.4		F62	34.1	
F5	30.6		F34	27.5		F63	33.3	
F6	35.5		F35	20.2	19.9	F64	30.3	
F7	29.6		F36	24.8	Neg	F65	31.8	
F8	37.5		F37	Neg		F66	33.5	
F9	32.0		F38	Neg		F67	31.7	
F10	28.6		F39	30.4		F68	30.4	
F11	32.2		F40	29.9		F69	34.2	
F12	31.6		F41	28.1		F70	33.4	
F13	32.8		F42	31.8		F71	32.7	
F14	31.5		F43	31.4		F72	31.0	
F15	33.9		F44	33.3		F73	32.7	
F16	30.4		F45	30.1		F74	34.0	
F17	26.9		F46	27.7		F75	33.0	
F18	30.9		F47	31.7		F76	32.2	
F19	33.8		F48	31.8		F77	33.0	
F20	32.2		F49	23.5	22.4	F78	33.3	
F21	30.6		F50	23.0	22.2	F79	32.6	
F22	32.6		F51	31.3		F80	31.4	
F23	31.0		F52	31.3		F81	30.3	
F24	35.1		F53	32.2		F82	31.8	
F25	30.0		F54	32.0		F83	33.0	
F26	32.5		F55	28.1		F84	32.9	
F27	33.0		F56	30.0		F85	33.4	
F28	35.0		F57	30.3				
F29	33.5		F58	32.3				

Table 22. Ct-values obtained from the site BO and the date of sampling.

Site: BO	Date: January 29	
Fish	PRV1M2	PRV1b
F1	24,7	19,6
F2	25,4	19,1
F3	24,5	19,3
F4	24,3	20,7
F5	25,2	17,5
F6	24,8	20,1
F7	24,0	21,2
F8	24,2	20,0
F9	21,4	16,7
F10	24,7	19,1

Table 23. Ct-values obtained from the site LH and the date of sampling.

Site: LH	Date: July 20	
Fish	PRV1M2	PRV1b
F1	20,4	19,9
F2	20,1	17,8
F3	21,3	20,2
F4	19,3	15,7
F5	17,9	16,5
F6	21,4	17,4
F7	18,4	17,2
F8	19,6	18,0
F9	17,3	15,0
F10	22,9	24,5

Table 24. Ct-values obtained from the site JV and the date of sampling.

Site: JV	Date: July 20	
Fish	PRV1M2	PRV1b
F1	20,5	16,0
F2	18,9	15,1
F3	19,2	16,1
F4	17,1	14,0
F5	18,3	14,4
F6	17,9	14,3
F7	19,2	15,4
F8	20,9	16,5
F9	18,6	14,5
F10	19,3	14,8

Table 25. Ct-values obtained from the site PV and the date of sampling.

Site: PV	Date: March 20	
Fish	PRV1M2	PRV1b
F1	24,4	22,6
F2	24,5	24,1
F3	22,7	19,9
F4	22,3	23,3
F5	21,4	19,1
F6	26,5	29,4
F7	21,1	18,4
F8	24,7	22,1
F9	18,1	15,7
F10	18,5	16,6

Table 26. Ct-values obtained from the site KN and the date of sampling.

Site: KN	Date: October 19	
Fish	PRV1M2	PRV1-A3
F1	24.9	20.5
F2	26.4	20.2
F3	26.4	23.3
F4	22.7	18.7

Table 27. Ct-values obtained from the site HV and the date of sampling.

Site: HV	Date: May 20	
Fish	PRV1M2	PRV1-A3
F1	19.3	17.1
F2	17.9	17.4
F3	16.4	16.7
F4	18.1	17.2
F5	18.2	16.7
F6	18.9	15.4
F7	17.5	16.1
F8	19.5	16.6
F9	18.7	15.0
F10	19.1	17.9

Table 28. Ct-values obtained from the site 2HV and the date of sampling.

Site: 2HV	Date: September 20	
Sampling	Fish	PRV1M2
0	F1	Neg
0	F2	Neg
0	F3	Neg
0	F4	Neg
0	F5	Neg
0	F6	Neg
0	F7	Neg
0	F8	Neg
0	F9	Neg
0	F10	Neg
0	F11	Neg
0	F12	Neg
0	F13	Neg
0	F14	Neg
0	F15	Neg
0	F16	Neg
0	F17	Neg
0	F18	Neg
0	F19	Neg
0	F20	Neg
0	F21	Neg
0	F22	Neg
0	F23	Neg
0	F24	Neg
0	F25	Neg
0	F26	Neg
0	F27	Neg
0	F28	Neg
0	F29	Neg
0	F30	Neg

Table 29. Ct-values obtained from the site 2HV and the date of sampling.

Site: 2HV				Date: September 20		
Sampling	Net pen	Fish	PRV1M2	Net pen	Fish	PRV1M2
1	SC	F1	Neg	O	F30	Neg
1	SC	F2	Neg	O	F32	Neg
1	SC	F3	Neg	O	F33	Neg
1	SC	F4	Neg	O	F34	Neg
1	SC	F5	Neg	O	F35	Neg
1	SC	F6	Neg	O	F36	Neg
1	SC	F7	Neg	O	F37	Neg
1	SC	F8	Neg	O	F38	Neg
1	SC	F9	Neg	O	F39	Neg
1	SC	F10	Neg	O	F40	Neg
1	SC	F11	Neg	O	F41	Neg
1	SC	F12	Neg	O	F42	Neg
1	SC	F13	Neg	O	F43	Neg
1	SC	F14	Neg	O	F44	Neg
1	SC	F15	Neg	O	F45	Neg
1	SC	F16	Neg	O	F46	Neg
1	SC	F17	Neg	O	F47	Neg
1	SC	F18	Neg	O	F48	Neg
1	SC	F19	Neg	O	F49	35.1
1	SC	F20	Neg	O	F50	Neg
1	SC	F21	Neg	O	F51	Neg
1	SC	F22	Neg	O	F52	Neg
1	SC	F23	Neg	O	F53	Neg
1	SC	F24	37.1	O	F54	Neg
1	SC	F25	Neg	O	F55	Neg
1	SC	F26	Neg	O	F56	Neg
1	SC	F27	Neg	O	F57	Neg
1	SC	F28	Neg	O	F58	Neg
1	SC	F29	Neg	O	F59	Neg
1				O	F60	Neg

Table 30. Ct-values obtained from the site 2HV and the date of sampling.

Site: 2HV			Date: September 20			
Sampling	Net pen	Fish	PRV1M2	Net pen	Fish	PRV1M2
2	SC	F1	Neg	O	F31	Neg
2	SC	F2	Neg	O	F32	Neg
2	SC	F3	Neg	O	F33	Neg
2	SC	F4	Neg	O	F34	Neg
2	SC	F5	Neg	O	F35	Neg
2	SC	F6	Neg	O	F36	Neg
2	SC	F7	Neg	O	F37	Neg
2	SC	F8	Neg	O	F38	Neg
2	SC	F9	Neg	O	F39	Neg
2	SC	F10	Neg	O	F40	Neg
2	SC	F11	Neg	O	F41	Neg
2	SC	F12	Neg	O	F42	Neg
2	SC	F13	Neg	O	F43	Neg
2	SC	F14	Neg	O	F44	Neg
2	SC	F15	Neg	O	F45	Neg
2	SC	F16	Neg	O	F46	Neg
2	SC	F17	Neg	O	F47	Neg
2	SC	F18	Neg	O	F48	Neg
2	SC	F19	Neg	O	F49	Neg
2	SC	F20	Neg	O	F50	Neg
2	SC	F21	Neg	O	F51	Neg
2	SC	F22	Neg	O	F52	Neg
2	SC	F23	Neg	O	F53	Neg
2	SC	F24	Neg	O	F54	Neg
2	SC	F25	Neg	O	F55	Neg
2	SC	F26	Neg	O	F56	Neg
2	SC	F27	Neg	O	F57	Neg
2	SC	F28	Neg	O	F58	Neg
2	SC	F29	Neg	O	F59	Neg
2	SC	F30	Neg	O	F60	Neg

Table 31. Ct-values obtained from the site 2HV and the date of sampling.

Site: 2HV			Date: September 20			
Sampling	Net pen	Fish	PRV1M2	Net pen	Fish	PRV1M2
3	SC	F1	Neg	O	F31	Neg
3	SC	F2	Neg	O	F32	Neg
3	SC	F3	Neg	O	F33	Neg
3	SC	F4	Neg	O	F34	Neg
3	SC	F5	Neg	O	F35	Neg
3	SC	F6	Neg	O	F36	Neg
3	SC	F7	Neg	O	F37	Neg
3	SC	F8	Neg	O	F38	Neg
3	SC	F9	Neg	O	F39	Neg
3	SC	F10	Neg	O	F40	Neg
3	SC	F11	Neg	O	F41	Neg
3	SC	F12	Neg	O	F42	Neg
3	SC	F13	Neg	O	F43	Neg
3	SC	F14	Neg	O	F44	Neg
3	SC	F15	Neg	O	F45	Neg
3	SC	F16	Neg	O	F46	Neg
3	SC	F17	Neg	O	F47	Neg
3	SC	F18	Neg	O	F48	Neg
3	SC	F19	Neg	O	F49	Neg
3	SC	F20	Neg	O	F50	Neg
3	SC	F21	Neg	O	F51	Neg
3	SC	F22	Neg	O	F52	Neg
3	SC	F23	Neg	O	F53	Neg
3	SC	F24	Neg	O	F54	Neg
3	SC	F25	Neg	O	F55	Neg
3	SC	F26	Neg	O	F56	Neg
3	SC	F27	Neg	O	F57	Neg
3	SC	F28	Neg	O	F58	Neg
3	SC	F29	Neg	O	F59	Neg
3	SC	F30	Neg	O	F60	Neg

Table 32. Ct-values obtained from the site 2HV and the date of sampling.

Site: 2HV				Date: October 20		
Sampling	Net pen	Fish	PRV1M2	Net pen	Fish	PRV1M2
4	SC	F1	Neg	O	F31	Neg
4	SC	F2	Neg	O	F32	Neg
4	SC	F3	Neg	O	F33	Neg
4	SC	F4	Neg	O	F34	Neg
4	SC	F5	Neg	O	F35	Neg
4	SC	F6	Neg	O	F36	Neg
4	SC	F7	Neg	O	F37	Neg
4	SC	F8	Neg	O	F38	Neg
4	SC	F9	Neg	O	F39	Neg
4	SC	F10	Neg	O	F40	Neg
4	SC	F11	Neg	O	F41	Neg
4	SC	F12	Neg	O	F42	Neg
4	SC	F13	Neg	O	F43	Neg
4	SC	F14	Neg	O	F44	Neg
4	SC	F15	Neg	O	F45	Neg
4	SC	F16	Neg	O	F46	Neg
4	SC	F17	Neg	O	F47	Neg
4	SC	F18	Neg	O	F48	Neg
4	SC	F19	Neg	O	F49	Neg
4	SC	F20	Neg	O	F50	Neg
4	SC	F21	Neg	O	F51	Neg
4	SC	F22	39.8	O	F52	Neg
4	SC	F23	Neg	O	F53	Neg
4	SC	F24	Neg	O	F54	Neg
4	SC	F25	Neg	O	F55	Neg
4	SC	F26	Neg	O	F56	Neg
4	SC	F27	Neg	O	F57	Neg
4	SC	F28	Neg	O	F58	Neg
4	SC	F29	Neg	O	F59	Neg
4	SC	F30	Neg	O	F60	Neg

Table 33. Ct-values obtained from the site 2HV and the date of sampling.

Site: 2HV				Date: October 20		
Sampling	Net pen	Fish	PRV1M2	Net pen	Fish	PRV1M2
5	SC	F1	Neg	O	F1	Neg
5	SC	F2	Neg	O	F2	Neg
5	SC	F3	Neg	O	F3	Neg
5	SC	F4	Neg	O	F4	Neg
5	SC	F5	Neg	O	F5	Neg
5	SC	F6	Neg	O	F6	Neg
5	SC	F7	Neg	O	F7	Neg
5	SC	F8	Neg	O	F8	Neg
5	SC	F9	Neg	O	F9	Neg
5	SC	F10	Neg	O	F10	Neg
5	SC	F11	Neg	O	F11	Neg
5	SC	F12	Neg	O	F12	Neg
5	SC	F13	Neg	O	F13	Neg
5	SC	F14	Neg	O	F14	Neg
5	SC	F15	Neg	O	F15	Neg
5	SC	F16	Neg	O	F16	Neg
5	SC	F17	Neg	O	F17	Neg
5	SC	F18	Neg	O	F18	Neg
5	SC	F19	Neg	O	F19	Neg
5	SC	F20	Neg	O	F20	Neg
5	SC	F21	Neg	O	F21	Neg
5	SC	F22	Neg	O	F22	Neg
5	SC	F23	Neg	O	F23	Neg
5	SC	F24	Neg	O	F24	Neg
5	SC	F25	Neg	O	F25	Neg
5	SC	F26	Neg			
5	SC	F27	Neg			
5	SC	F28	Neg			
5	SC	F29	Neg			
5	SC	F30	Neg			

Table 34. Ct-values obtained from the site 2HV and the date of sampling.

Site: 2HV				Date: November 20		
Sampling	Net pen	Fish	PRV1M2	Net pen	Fish	PRV1M2
6	SC	F1	Neg	O	F31	Neg
6	SC	F2	Neg	O	F32	Neg
6	SC	F3	Neg	O	F33	Neg
6	SC	F4	Neg	O	F34	Neg
6	SC	F5	Neg	O	F35	Neg
6	SC	F6	Neg	O	F36	Neg
6	SC	F7	Neg	O	F37	Neg
6	SC	F8	Neg	O	F38	Neg
6	SC	F9	Neg	O	F39	Neg
6	SC	F10	Neg	O	F40	Neg
6	SC	F11	Neg	O	F41	Neg
6	SC	F12	Neg	O	F42	Neg
6	SC	F13	Neg	O	F43	Neg
6	SC	F14	Neg	O	F44	Neg
6	SC	F15	Neg	O	F45	Neg
6	SC	F16	Neg	O	F46	Neg
6	SC	F17	Neg	O	F47	Neg
6	SC	F18	Neg	O	F48	Neg
6	SC	F19	Neg	O	F49	Neg
6	SC	F20	Neg	O	F50	Neg
6	SC	F21	Neg	O	F51	Neg
6	SC	F22	Neg	O	F52	Neg
6	SC	F23	Neg	O	F53	Neg
6	SC	F24	Neg	O	F54	Neg
6	SC	F25	Neg	O	F55	Neg
6	SC	F26	Neg	O	F56	Neg
6	SC	F27	Neg	O	F57	Neg
6	SC	F28	Neg	O	F58	Neg
6	SC	F29	Neg	O	F59	Neg
6	SC	F30	Neg	O	F60	Neg

Table 35. Ct-values obtained from the site 2HV and the date of sampling.

Site: 2HV			Date: Desember 20			
Sampling	Net pen	Fish	PRV1M2	Net pen	Fish	PRV1M2
7	SC	F1	Neg	O	F31	Neg
7	SC	F2	Neg	O	F32	Neg
7	SC	F3	Neg	O	F33	Neg
7	SC	F4	Neg	O	F34	39.1
7	SC	F5	Neg	O	F35	Neg
7	SC	F6	Neg	O	F36	Neg
7	SC	F7	Neg	O	F37	Neg
7	SC	F8	Neg	O	F38	Neg
7	SC	F9	23.3	O	F39	Neg
7	SC	F10	38.0	O	F40	Neg
7	SC	F11	Neg	O	F41	Neg
7	SC	F12	Neg	O	F42	Neg
7	SC	F13	Neg	O	F43	Neg
7	SC	F14	Neg	O	F44	Neg
7	SC	F15	Neg	O	F45	Neg
7	SC	F16	Neg	O	F46	Neg
7	SC	F17	Neg	O	F47	Neg
7	SC	F18	Neg	O	F48	Neg
7	SC	F19	Neg	O	F49	Neg
7	SC	F20	Neg	O	F50	Neg
7	SC	F21	Neg	O	F51	Neg
7	SC	F22	Neg	O	F52	39.9
7	SC	F23	Neg	O	F53	Neg
7	SC	F24	Neg	O	F54	Neg
7	SC	F25	Neg	O	F55	Neg
7	SC	F26	Neg	O	F56	39.6
7	SC	F27	Neg	O	F57	Neg
7	SC	F28	Neg	O	F58	Neg
7	SC	F29	Neg	O	F59	Neg
7	SC	F30	Neg	O	F60	Neg

Table 36. Ct-values obtained from the site 2HV and the date of sampling.

Site: 2HV			Date: January 21			
Sampling	Net pen	Fish	PRV1M2	Net pen	Fish	PRV1M2
8	SC	F1	Neg	O	F31	Neg
8	SC	F2	Neg	O	F32	Neg
8	SC	F3	Neg	O	F33	Neg
8	SC	F4	17.1 - seq	O	F34	Neg
8	SC	F5	35.0	O	F35	Neg
8	SC	F6	34.5	O	F36	Neg
8	SC	F7	Neg	O	F37	Neg
8	SC	F8	Neg	O	F38	Neg
8	SC	F9	Neg	O	F39	Neg
8	SC	F10	Neg	O	F40	Neg
8	SC	F11	Neg	O	F41	Neg
8	SC	F12	Neg	O	F42	Neg
8	SC	F13	Neg	O	F43	Neg
8	SC	F14	Neg	O	F44	Neg
8	SC	F15	Neg	O	F45	Neg
8	SC	F16	Neg	O	F46	Neg
8	SC	F17	Neg	O	F47	Neg
8	SC	F18	Neg	O	F48	Neg
8	SC	F19	Neg	O	F49	Neg
8	SC	F20	Neg	O	F50	21.9 - seq
8	SC	F21	Neg	O	F51	Neg
8	SC	F22	Neg	O	F52	Neg
8	SC	F23	33.6	O	F53	39.6
8	SC	F24	34.7	O	F54	Neg
8	SC	F25	Neg	O	F55	Neg
8	SC	F26	Neg	O	F56	Neg
8	SC	F27	Neg	O	F57	Neg
8	SC	F28	Neg	O	F58	Neg
8	SC	F29	38.1	O	F59	Neg
8	SC	F30	Neg	O	F60	Neg

Table 37. Ct-values obtained from the site HM and the date of sampling.

Site: HM	Date: October 20	
Fish	PRV1M2	PRV1-A3
F1	23.6	18.6
F2	19.3	13.3
F3	25.0	19.4
F4	21.1	15.9
F5	25.7	21.4
F6	23.5	17.9

Table 38. Ct-values obtained from the site VF and the date of sampling.

Site: VF	Date: April 20	
Fish	PRV1M2	PRV1-A3
F1	22.6	21.6
F2	24.2	21.0
F3	19.7	17.4
F4	22.4	24.0
F5	23.0	19.8
F6	22.2	19.9

Table 39. Ct-values obtained from the site HO and the date of sampling.

Site: HO	Date: October 20	
Fish	PRV1M2	PRV1-A3
F1	20.7	17.9
F2	20.3	17.4
F3	20.6	17.4
F4	19.9	13.9
F5	19.7	23.6
F6	19.9	16.7
F7	18.7	15.6
F8	19.9	16.7
F9	19.4	16.0
F10	20.6	17.6