Tissue tropism and optimization and evaluation of diagnostics test for the detection of Pilchard orthomyxovirus (POMV) in Atlantic salmon (*Salmo salar*)

Thesis for the degree

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Abbreviations

cDNA	Complementary DNA
CHSE	Chinook salmon embryo cells
CPE	Cytopathic effect
Ct-value	Cycle threshold-value
Dpi	Days post injection
ELF1α	Elongation factor 1 alpha
HPR0	Low virulent ISAV
HPRΔ	High virulent ISAV
LOD	Limit of detection
IAV	Influenza A virus
IHC	Immunohistochemistry
ISAV	Infectious salmon anaemia virus
IP-injection	Intraperitoneal injection
mRNA	Messenger ribonucleic acid
nM	Nanomolar
ORF	Open reading frame
OIE	World Organization for Animal Health
POMV	Pilchard orthomyxovirus
RT-qPCR	Quantitative reverse transcription PCR
RbtOV	Rainbow trout orthomyxovirus
SON	Salmon orthomyxoviral necrosis
µl	Microliter

Abstract

Since 2012, pilchard orthomyxovirus (POMV) has been of major concern to the Tasmanian salmonid industry. The virus was first discovered in pilchards, and is, so far, only shown to be highly infectious to Atlantic salmon (*Salmo salar*). Salmonid orthomyxovirus necrosis (SON) is caused by POMV and is associated with multisystemic pathology causing high mortalities in the marine phase. The aims of this study were to investigate the tissue tropism and to develop a new diagnostic test to improve early detection of POMV in Atlantic salmon. For this, tissue samples of sub-clinical smolt from a cohabitation challenge were examined. POMV was detected, post injection, in all six tissues examined with a peak of infection between 8- and 12-days in all organs of sub-clinical fish with the exception of the gills which presented high viral loads past this point. Head kidney and heart were the first sites to show presence of POMV in sub-clinical fish, two days post injection, and were also among the tissues with highest viral loads throughout the time course of the experiment. This knowledge revealed that these tissues are effective samples for POMV detection. RT-qPCR assays targeting segment 7 and 8 of the genome of POMV were designed and validated in several steps. However, the improved diagnostic sensitivity of the assays compared to the currently used RT-qPCR assay targeting segment 5, especially for the segment 8 assay, will be of particularly importance to contribute to earlier detection of the virus. Development of an improved diagnostic tool for early detection of POMV is of importance and will allow management of disease at earlier phases, improve animal welfare, and potentially reduce prevalence, transmission and losses due to the virus.

Introduction

1.1 Introduction to salmonid farming in Tasmania

Commercial salmonid farming commenced in Australia in the mid-1980s and, is today, mainly composed of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) (Llewellyn, 2015; Mobsby, 2018). The salmon industry mostly operates in Tasmania in sea pens (98%) and only a small quantity of salmonids are produced in Victoria and New south Wales in land-based ponds (2%) (CSIRO 2016; Mobsby, Steven and Curtotti, 2020). Production of salmonids (mainly Atlantic salmon) has increased from 40,405 tonnes in 2013-2014 to 61,033 tonnes in 2017-2018 (Fig. 1), and the three major Tasmanian companies are; Tassal Group Ltd (Tassal), Huon Aquaculture Group and Petuna Pty Ltd (Huon Aquaculture, 2016, 2018; Mobsby, D and Koduah, 2017; Mobsby et al., 2020; Mosby, 2018; Tassal, 2013, 2018). Internationally Australia accounted for about 2% the global salmonid harvest (Everblu Research, 2018). However, domestically the salmonid industry accounted for 56% of the Australian aquaculture (tonne of product), followed by oyster (13%), tuna (9%), and prawns (5%) (etc), representing a value of AUD 756 million in 2016- 2017 (Australian Fisheries and Aquaculture Statistics 2018, 2020).

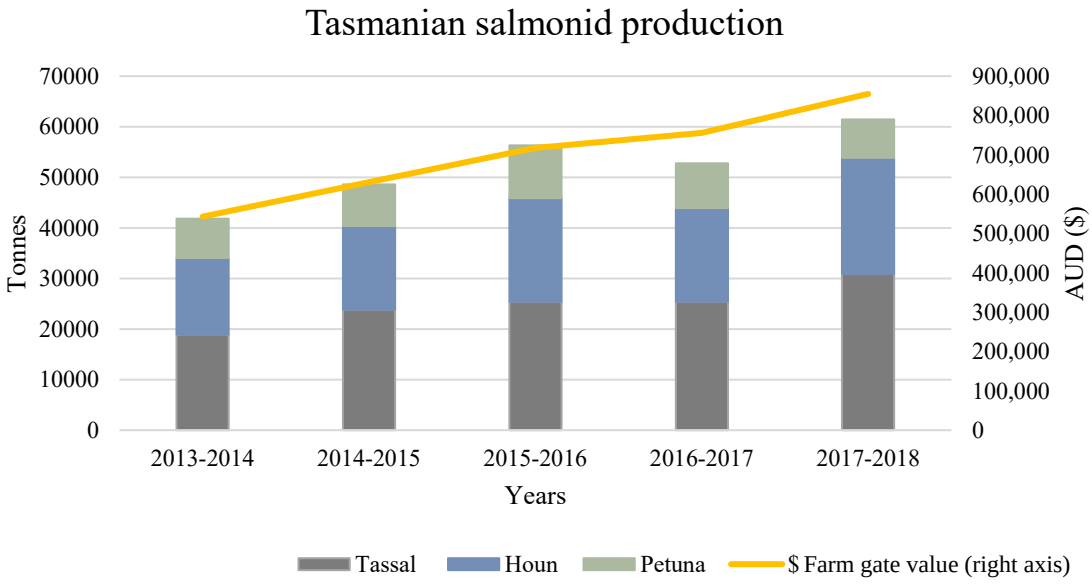


Figure 1. Tasmanian salmonid production by company (Tassal, Houn and Petuna) from 2013-2014 to 2017-2018. Note: Petuna do not report volumes and were calculated as ABARES total production estimate less reported totals for Tassal and Huon (Huon Aquaculture, 2016, 2018; Mobsby et al, 2017; Mobsby et al., 2020; Mosby, 2018; Tassal, 2013, 2018).

Salmonids are native to the northern hemisphere and were introduced to Australia in the late 1800s for recreational angling. In Tasmania there is presently a self-recruiting population of rainbow trout, brown trout (*Salmo trutta*) and small numbers of brook trout (*Salvelinus fontinalis*) in lakes and rivers (Cadwallader, 1996). All stocks of Atlantic salmon and brook trout descent from Canada (Nova Scotia), and Rainbow trout from the north west coast of America (Cadwallader, 1996). Import took place several times until the 1960s when imports were banned, as it presented a risk of introduction of exotic disease to Australia (Llewellyn, 2015). For the very same reason, today, only "consumer-ready" salmon products can be imported into Australia (with the exception of Tasmania) (CSIRO, 2016).

The marine grow-out phase takes place in open pens, each containing between 60.000 to 200.000 fish, located in mainly two areas in Tasmania. The *south-east region* in proximity to the D'Entrecasteaux Channel, Huon River, Port Esperance, Stormy bay, Tasman peninsula and one farm in Okehampton Bay. In *the north-west region* in Macquarie harbor, in addition to one farm in the north of the state, located in the Tamar estuary (Fig. 2) (DPWE, 2016). Tasmanian salmonid farming is conducted in both the ocean and in estuaries, which have different water salinities and water exchange conditions, and therefore different problems and diseases associated (DPWE, 2016).

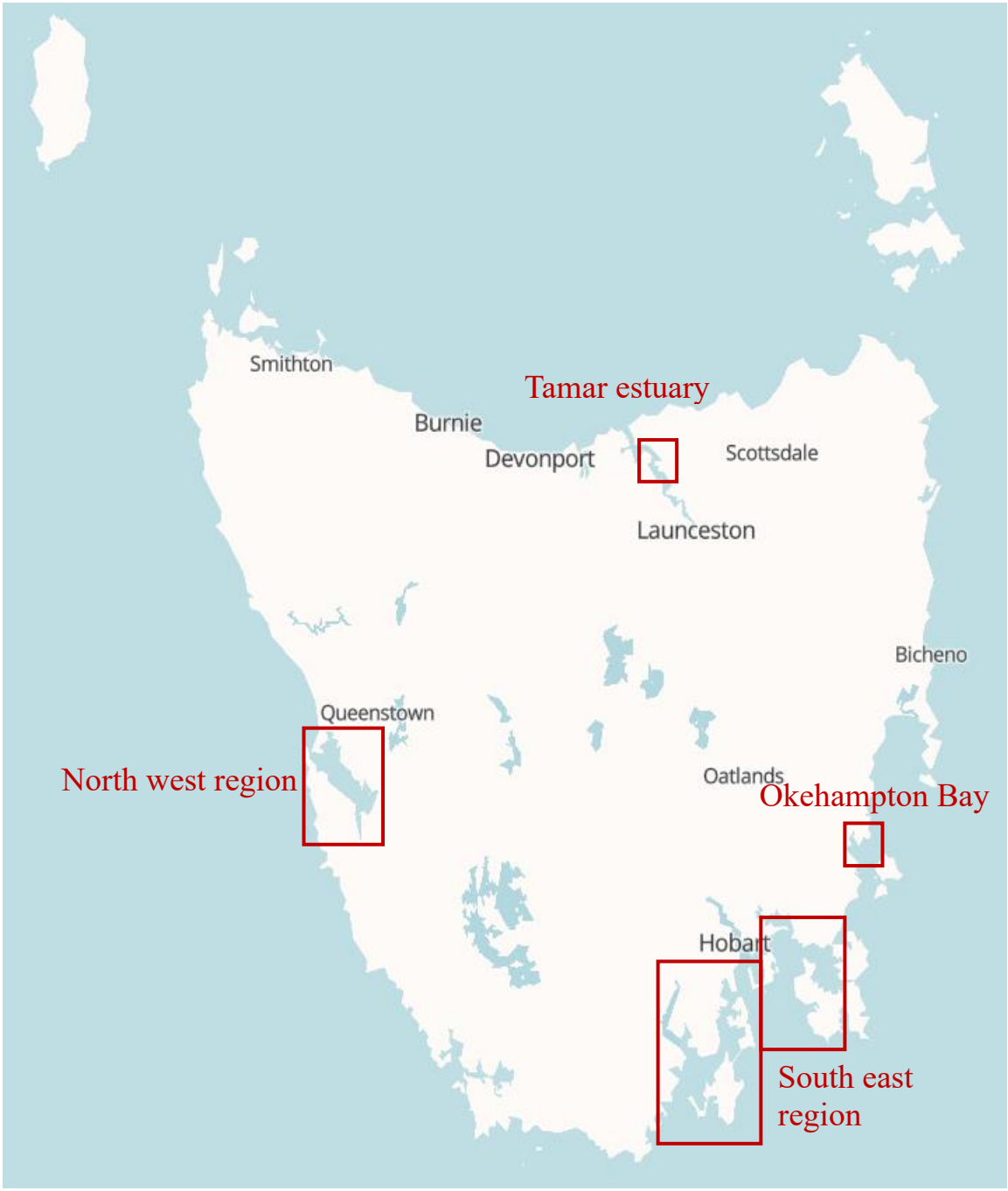


Figure 2. The current fin fish production areas in Tasmania marked in red, adapted from (DPWE, 2016).

Most fin fish diseases listed as reportable by OIE are exotic to Australia, and the list includes none of the diseases of concern for the Tasmanian salmonid industry (Animal Health Committee 2018). The main endemic diseases are; amoebic gill disease (AGD, *Paramoeba perurans*), salmonid orthomyxovirus necrosis (SON, *Pilchard orthomyxovirus*), yersiniosis (*Yersinia ruckeri* serotype O1b), vibriosis (*Vibrio anguillarum* serotype O1), *Tenacibaculum maritimum*, marine *Flavobacterium* spp., Tasmanian Aquareovirus (TSRV) and Tasmanian rickettsia-like organism (RLO). In addition, the Tasmanian aquabirnavirus and atypical

furunkulosis (*Aeromonas salmonicida*) which is unique for Macquarie harbor (Huon Aquaculture, 2017; Animal Health Committee, 2018) are of concern. *Paramoeba perurans* cause infection in the gills and AGD has been the primary health problem from the beginning of the Tasmanian salmonid industry. The disease is associated with high mortalities (up to 50% if untreated), and is expensive to control and treat (Munday et al., 1990; Taylor et al., 2009). SON, however, was introduced to the Tasmanian industry in the recent past and is now an established and serious disease affecting the salmon industry (Samsing et al., *in press*.; Godwin et al., 2020; Godwin et al. 2016; Morrison et al., 2013).

1.2 History of POMV

A novel orthomyxo-like virus was detected in wild pilchards (*Sardinops sagax*) in South Australia in 1998, as an incidental finding during investigation of mass mortality events caused by a pilchard herpesvirus (PHV) (Godwin et al., 2016; Hyatt et al., 1997; Whittington et al., 1997). The virus was named pilchard orthomyxovirus (POMV) (Godwin et al., 2016). In 2006 another orthomyxo-like virus with similar virus morphology was isolated from apparently healthy Atlantic salmon during routine health surveillance testing in Tasmania's Tamar River. The new virus was named salmon orthomyxovirus (SOMV), and both viruses were confirmed not to be infectious salmon anaemia virus (ISAV), a serious disease of Atlantic salmon, exotic to Australia (Department of Agriculture, 2019; Godwin et al., 2016). In 2012, SOMV was shown to be pathological as the virus was obtained from diseased Atlantic salmon in an outbreak in south east Tasmania with high mortalities (> 500 000 fish) (Godwin et al., 2016; Huon Aquaculture, 2017). Recently, POMV and SOMV were confirmed to be the same virus (>95% nucleotide sequence identity) and has thereafter been named POMV (Mohr et al., 2020). The disease caused by POMV was named salmon orthomyxoviral necrosis (SON) (Godwin et al., 2020). Since 2012 the virus has been detected in all production areas in Tasmania and SON has shown to cause up to 50% mortality in individual pens (Godwin et al., 2016; Huon Aquaculture, 2017). SON is a serious disease and has the potential to severely impact the Tasmanian salmon industry (Huon Aquaculture, 2017).

1.3 Orthomyxovirus classification and virion properties

POMV is a member of the *Orthomyxoviridae* family (Mohr et al.,2020), a family of enveloped, negative sense, single stranded RNA viruses where the genome consists of six to eight linear segments. The orthomyxovirus virions are about 80-100 nm, spherical or pleomorphic and their genome size ranges from 10.0 to 14.6 kb encoding up to 12 proteins (Cler et al., 1983; Lamb & Krug, 1996; McCauley et al., 2012; McGeoch et al., 1976; Mjaaland et al., 1997). *Orthomyxoviridae* consists of seven genera: *Alphainfluenzavirus* (Type species: *Influenzavirus A), Betainfluenzavirus* (Type species*: Influenzavirus B), Gammainfluenzavirus* (Type species*: Influenzavirus C), Deltainfluenzavirus* (Type species*: Influenzavirus D), Thogotovirus* (Type species: *Thogoto thogotovirus), Isavirus* (Type species: *Salmon Isavirus*) and *Quaranjavirus* (Type species: *Quaranfil quaranjavirus)* (International Committee on the Taxonomy of Viruses, 2020) The prototype of the family, Influenza A (IAV), is relevant to the marine environment as its main reservoir is aquatic birds (Webster et al., 1992). Currently, the only fish virus in the orthomyxovirus family is the *Infectious salmon anaemia virus* (ISAV) (Falk et al., 1997; Koren & Nylund, 1997; Krossøy et al., 1999; Mjaaland et al., 1997).

ISAV is one of the most important viruses in the global salmonid aquaculture industry, consisting of two major genotypes (North American and European) (Devold et al., 2006; A Nylund et al., 2007; Plarre et al., 2012; Vike, 2014), causing disease in farmed Atlantic salmon in Norway (Thorud & Djupvik, 1988), Canada (Lovely et al., 1999; Mullins et al., 1998; Ritchie et al., 2001a), USA (Bouchard et al., 2001), Faroe Islands (Lyngøy, 2003), Scotland (Rodger et al., 1998; Rowley et al., 1999), Ireland (Nylund et al., 2007) and Chile (Godoy et al., 2008; Kibenge et al., 2001). However, recently a new orthomyxovirus was detected in USA, rainbow trout orthomyxovirus (RbtOV), in Rainbow trout (Batts et al., 2017). Based on phylogenetic studies, RbtOV and POMV are both likely to represent two new genera within the *Orthomyxoviridae* family, with their closest relative being ISAV (Batts et al., 2017; Godwin et al., 2016). Polymerase basic protein 1 (PB1) is the most conserved protein of the orthomyxoviruses (Leahy et al., 1997; Lin et al., 1991; Nuttall et al., 1995; Yamashita et al., 1989) and therefore used in these studies to evaluate the evolutionary relationship between orthomyxoviruses. PB1 share a 40% similarity between POMV and ISAV, while the fusion protein (F) shows the lowest similarity, 23% between POMV and ISAV (Godwin et al., 2016; Mohr et al., 2020).

IAV, ISAV, RbtOV and POMV all have a genome around 13500 nucleotides in length and are composed of 8 segments (Table 1). Proteins of these orthomyxoviruses are orthologues and share multiple proteins and functional domains, with some variation in the size of the viral segments encoding for each protein, and the number of proteins encoded.

Table 1. The different segments ranked by size and the protein encoded by them for POMV, ISAV, RbtOV, IAV. The protein abbreviations are: PB2= polymerase basic protein 2, PB1= polymerase basic protein 1, NP= nucleocapsid protein, PA= acidic polymerase protein, NA= neuraminidase, F=fusion protein, HA= hemagglutinin, HE= hemagglutinin esterase protein, s8ORF 2/Non-structural protein 1(NS1)= IFN antagonists, NEP= nuclear export protein, M1= matrix protein 1 and M2= matrix protein 2.

Ranked by size	**POMV** [a]	**ISAV**	**RbtOV** [h]	**IAV** [i]
1	PB2	PB2 [b]	PB2	PB2
2	PB1	PB1 [c]	PB1	PB1
3	NP	NP [d]	NP	PA
4	F/S4B	PA [d]	PA	HA
5	PA	F [e]	NA	NP
6	HE	HE [f]	HA	NA
7	S7Xa S7Xb	NS1 /NEP [g]	NS1/NEP	M1/M2
8	8ORF	M1/s8ORF2 [g]	M1/M2	NS1/NEP

References: a= (Mohr et al., 2020), b= (Snow et al., 2003) c= (Krossøy et al., 1999) d= (Aspehaug et al., 2004; Falk et al., 2004; Goić et al., 2008; Ritchie et al., 2001b) e= (Aspehaug et al., 2004, 2005; Devold et al., 2006) f=(Falk et al., 2004; Kristiansen et al., 2002; Krossøy et al., 2001a, 2001b; Müller et al., 2010) g= (Biering et al., 2002; Falk et al., 2004; García-Rosado et al., 2008; Kibenge et al., 2007; Li et al., 2016; Mcbeath et al., 2006) h= (Batts et al. 2017) i=(Lamb & Krug, 1996; McGeoch et al., 1976)

Generally, orthomyxoviruses have one to two surface glycoproteins incorporated in the envelope: hemagglutinin esterase protein (HE)/F protein for ISAV and POMV (Aspehaug et al., 2005; Falk et al., 2004; Mohr et al., 2020), and hemagglutinin protein (HA)/neuraminidase protein (NA) for IAV and RbtOV (Batts et al., 2017; Lamb & Krug, 2001). Three biological

activities are related to these surface proteins: hemagglutination (receptor recognition) (Falk et al., 1997), esterase (receptor destruction) (Falk et al., 1997, 2004; Hellebo et al., 2004) and (F protein) fusion activity (Aspehaug et al., 2005; Eliassen et al., 2000). In ISAV and POMV, hemagglutination and esterase activities are located on the same individual protein, HE protein (Falk et al., 2004), while fusion activity is related to the F protein (Aspehaug et al., 2005). In IAV and RbtOV the hemagglutination activity is located on HA protein together with the fusion activity, while the neuraminidase activity is related to NA protein. Additionally, IAV and RbtOV have an ion channel located in the envelope, matrix protein 2 (M2) (R. Lamb & Krug, 2001). Under the envelope the matrix 1 proteins form a coat, which is associated with both the tails of the surface proteins and the 8 ribonucleoproteins (RNPs) (Aspehaug et al., 2004; Biering et al., 2002; Falk et al., 2004). The fourth major protein of ISAV, nucleoprotein, form the RNPs with RNA-dependent RNA polymerase complex (RdRp) composed of the proteins: polymerase basic protein 2 (PB2), PB1 and acidic polymerase (PA) and negative stranded ssRNA (Aspehaug et al., 2004; Falk et al., 2004). Nuclear export protein (NEP) is also associated with the nucleoprotein, and together with the RdRp proteins make up the minor structural proteins (Kibenge et al., 2007) (Fig. 3). The interferon antagonists, s8ORF2 and NS1, are non-structural proteins which suppress immune responses in the host (Biering et al., 2002; Mcbeath et al., 2006; García-Rosado et al., 2008; Li et al., 2016).

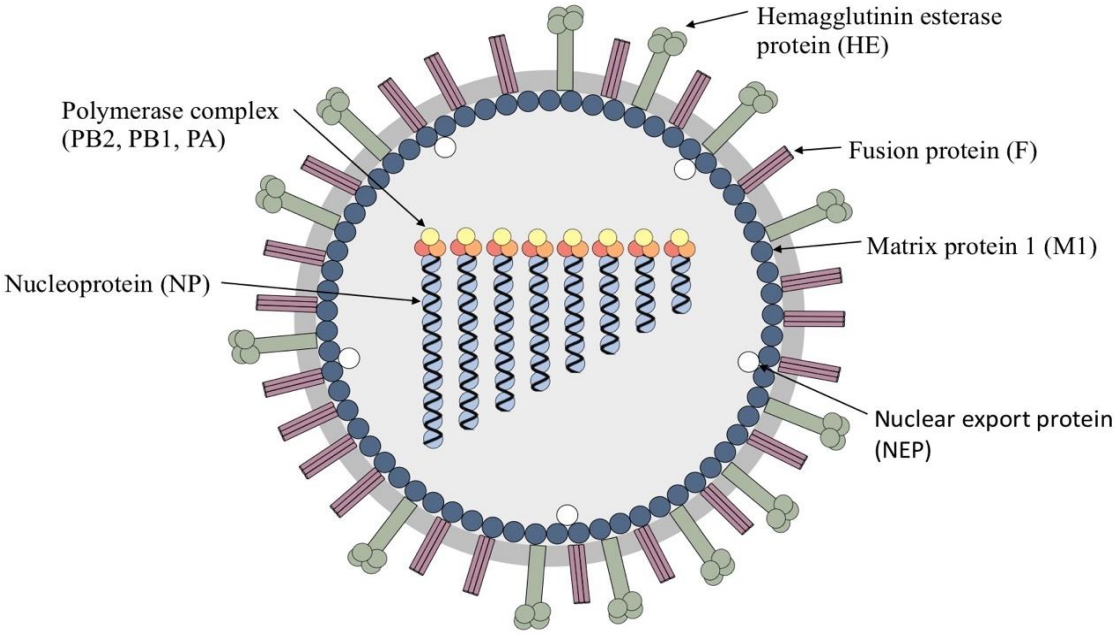


Figure 3. Schematic representation of pilchard orthomyxovirus (POMV) which includes the structural proteins and the 8 segments of the genome of the virion (adapted from Cottet et al., 2011)

The ISAV genome encodes 10 proteins (Mjaaland et al., 1997), six of these are homologs between POMV and ISAV, with some variation in size of the corresponding segment (Table 1). All of these six segments have one open reading frame (ORF) except for segment 4 of POMV. Segment 4 has a potential additional ORF, although the encoded protein has no matches with other protein of the orthomyxoviruses. Segment 7 of POMV has two possible ORF and may encode two proteins similar to segment 7 of ISAV. Segment 8 has been found to only have one ORF unlike ISAV which has two ORF (Table. 1). The possible encoded proteins of segment 7 and 8 of POMV do not align with proteins of ISAV segment 7 or 8, or those of other orthomyxoviruses (Mohr et al., 2020).

1.4 Replication

The first step in the viral replication for orthomyxoviruses is virus attachment by binding of hemagglutinin (HE or HA) to sialic acid on the cell's surface, and the virion enters the cell by receptor-mediated endocytosis (Hellebo et al., 2004; Kristiansen et al., 2002; Krossøy et al., 2001a; Rimstad et al., 2011; Workenhe et al., 2007) (Fig. 4a/b). A decrease in pH, involving M2 for IAV (R. Lamb & Krug, 2001), induces release of vRNP from M proteins and uncoating by fusion (F or HA) of the envelope with the endosome (Aspehaug et al., 2005; Eliassen et al., 2000) (Fig. 4c). This allows vRNPs into the cytoplasm which are then actively transported by importins (produced by the cell) into the cell nucleus (Wu et al., 2007) (Fig 4d). Unique for orthomyxoviruses are that they replicate inside the nucleus and are able to access capped cellular mRNA and exploit the host splicing machinery, unlike most other RNA viruses which remain in the cytoplasm (Falk et al., 1997; Palese, 2007).

The negative stranded RNA genome is not a part of the central dogma and cannot be used as a template of the cell's transcriptional machinery. The genome is transcribed by the virus´ own RdRp-complex: PB2 bind to the host pre-mRNA, PA cleaves it (referred to as "cap snatching") and the "cap" is used as a primer for the PB1 to initiate elongation (Dias et al., 2009; Sandvik et al., 2000). Early proteins, PB1, PB2, PA, NP and IFN antagonist, are transcribed first as these are essential for further synesis, followed by late proteins: HE/HA, F/NA, M1, M2 and NEP (Palese, 2007) (Fig 4e). The RdPd-complex (PB1, PB2 and PA) enables further translation and replication, NP stabilize RNA and is essential for the RdPd to bind to RNA and they are therefore transported into the nucleus (Aspehaug et al., 2004; Goić et al., 2008; Portela & Digard, 2002). Segments with two ORFs generates mRNA by

transcription of two alternative ORFs or through splicing resulting in an alternative ORF (Biering et al., 2002; García-Rosado et al., 2008; Kibenge et al., 2007; Mcbeath et al., 2006; Palese, 2007).

The mRNAs are transported to the cytoplasm for translation: the surface proteins on ribosomes associated with endoplasmic reticulum (ER) and the others on free ribosomes (Fig 4 f,g,h). In ER the proteins are modified, before entering the golgi apparatus (Falk et al., 2004; Palese, 2007). The surface proteins are transported to the cell surface by golgi, and the fusion related protein (F/HA) are activated by proteolytic cleave (Aspehaug et al., 2005; Falk et al., 2004;Lamb & Krug, 1996; Müller et al., 2010). Replication of vRNA also occurs in the nucleus to complimentary RNA (cRNA) followed by transcription of new viral RNA (vRNA). NP, M1 and NEP are imported into by importins to the nucleus to form an "export complex" which is essential for export of RNA form the nucleus to the cytoplasm. RNPs are actively transported out of the nucleus through exportins by recognition of NEP (Nayak et al., 2009) (Fig. 4i). These proteins are then incorporated with the surface protein on the cells' membrane by a budding process (Maria Aamelfot et al., 2012; Koren & Nylund, 1997). The virion is released from the cell by esterase (HE and HA) cuts sialic acid (Falk et al., 1997, 2004; Hellebo et al., 2004; Palese, 2007)(Fig. 4 j,k).

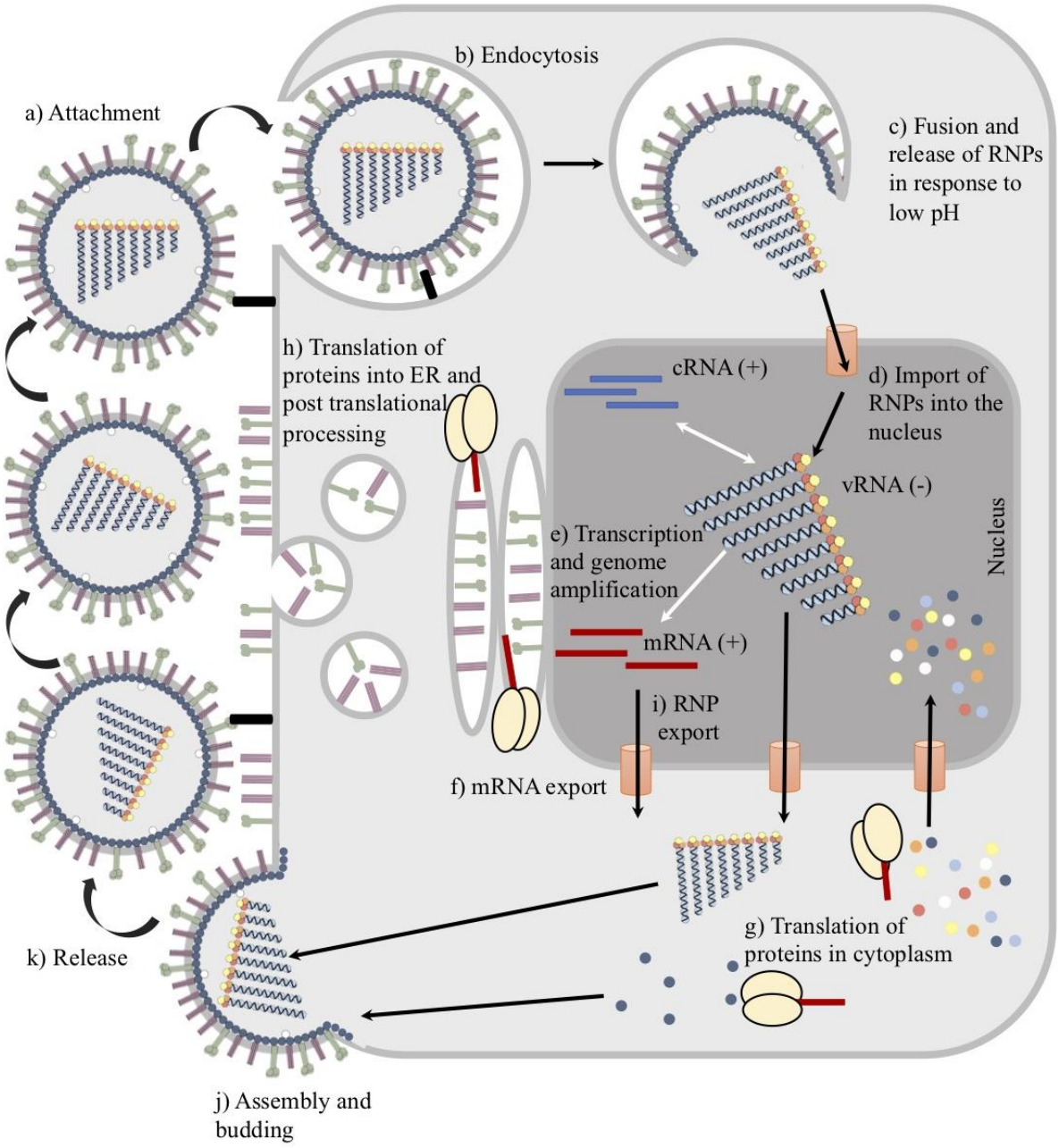


Figure 4. The replication cycle of orthomyxoviruses illustrated (adapted from Aspehaug, 2005).

1.5 Pathology, histopathology and cell/tissue tropism

Outbreaks of salmon orthomyxoviral necrosis (SON) has only been detected in Atlantic salmon in sea water (Godwin et al., 2016). Pathology associated with POMV in Atlantic salmon include lethargic fish seen swimming near the surface with dark coloration of the skin and petechial haemorrhages on ventral part of the body. Internally, clinical signs of disease include clear/opaque mucus in the stomach and gastrointestinal tract, splenomegaly, petechiae of visceral fat and peritoneal surfaces (Samsing et al., *in press*.; Godwin et al., 2020; Godwin

et al. 2016; Morrison et al., 2013). The presence of necrotic cells in multiple organs is the most constant observed change associated with POMV infection, given rise to the name of the disease; salmon orthomyxoviral necrosis (Godwin et al., 2020). Histologically, necrosis is observed in liver, kidney, spleen, heart and eye. These include mild multifocal hepatocellular necrosis, necrosis of renal haematopoietic tissue, inflammation and necrosis of spleen, necrosing mycarditis and uveitis (Samsing et al., *in press*; Godwin et al., 2020; Godwin et al. 2016). Usually individual fish display only a few of the clinical pathology associated with SON. Individually these changes are not exclusive for SON and is also associated with other pathogens of Atlantic salmon in Tasmania (Godwin et al., 2020). SON and ISA share pathology signs related to circulatory failure (Evensen et al., 1991; Thorud & Djupvik, 1988). However, anaemia, a characteristic feature of ISA, is not associated with POMV infection (Evensen, Thorud, and Olsen 1991; Godwin et al. 2020; Morrison, Carson, and Knowles 2013; Thorud and Djupvik 1988).

The cellular tropism of POMV is unknown and several cells are suggested to be target, however a particular tropism towards endothelial cells has been described (Samsing et al., *in press*; Godwin et al. 2020; Morrison et al. 2013). Similarly, other orthomyxoviruses are known to target endothelial cells, including ISAV and highly pathogenic IAV (Hovland et al 1994; Nylund et al 1995b; Koren & Nylund 1997; Nylund et al 1996, 1997; Aamelfot et al. 2012; Subbarao et al. 1998). Infection in endothelial cells can degenerate blood vessels and lead to haemorrhages in multiple organs and reduced fluid balance (Koren & Nylund 1997; Aamelfot et al. 2012). It is therefore likely that infection of endothelial cells by POMV is related to the vascular disturbances and haemorrhages associated with SON and might explain the multi tissue pathology (Godwin et al., 2020). Hepatocytes has also been suggested to be targeted (Godwin et al., 2020; Morrison et al. 2013), which correlates with the pathology observed in the liver with necrosis of individual hepatocytes (Godwin et al., 2020; Godwin et al. 2016; Morrison et al. 2013). In contrast to the characteristic pattern of necrosis seen for ISAV; hepatocyte necrosis in some distance from sinusoidal vessels while the tissue in close proximity of the central veins is intact (Evensen et al., 1991; Thorud & Djupvik, 1988). Among the target cells proposed, spleenocytes and haemopoetic cells in kidney also recur as potential targets (Samsing et al., *in press*; Morrison et al. 2013) which might explain the pathology seen in these organs.

POMV is shown to agglutinate erythrocytes and exhibit a receptor destroying activity, typical characteristics of orthomyxoviruses (Nylund et al 1995b; Koren & Nylund 1997; Hellebø et al 2004; Aamelfot 2013; Godwin et al., 2020; Godwin et al. 2016). Unlike the other target cells for ISAV (epithelial cells, endothelial cells, endocardial cells and leucocytes) (Dannevig et al., 1995; Hovland et al., 1994; Nylund et al., 1995b, 1996) there is no replication in the erythrocytes and the virus only binds to the surface of the cells (Aamelfot et al., 2012). This trait is known to cause agglutination of erythrocytes leading to reduced circulation flow. It has also been suggested that erythrocytes attachment simulates hemophagocytes as the immune system recognize them as forging, leading to blood cell depletion and anaemia for ISAV (Aamelfot et al., 2012). However, anaemia is not associated with SON, and the implications of viral attachment is not clear (Godwin et al., 2020). The vascular disturbances in addition to reduction of red blood cells have also been proposed to cause hypoxia which plausibly, indirectly cause necrotic lesions seen for ISAV (eg. seen for liver) (Aamelfot et al., 2012). Hypoxia is not a described for SON, and the cause of the necrotic pathology must be investigated further.

1.6 Host range

POMV was first discovered in apparently healthy pilchards (*Sardinops sagax*) and a few years later the virus was detected in diseased Atlantic salmon. These are the only species the virus has been isolated from, possibly demonstrating an interspecies transmission, well documented for other pilchard orthomyxoviruses (Mohr et al, 2020, McCauley et al., 2012). Despite being able to replicate in cell lines from both pilchard and salmon origin (Mohr et al, 2020), no evidence of disease has been observed in infected pilchards (Godwin et al., 2020; Godwin et al. 2016). Sequence analysis indicate that pilchards can act as a biological vector as the virus is shown to be present in both species at the same time (Godwin et al., 2016). Screening of other wild fish has not been conducted, and it is not known if other species then pilchards that are susceptible to POMV infection (Godwin et al., 2020.; Godwin et al. 2016).

POMV has only been shown to cause disease in Atlantic salmon in the field (Godwin et al., 2020). Experimentally, the virus has been shown to be pathogenic in both fresh water and seawater, and to all major life stages of Atlantic salmon (Godwin et al., 2020). Similarly, ISAV has only caused outbreaks in Atlantic salmon, almost exclusively detected in the seawater phase, and known to be susceptible to ISAV for all major life stages (Rimstad et al.

2002). Experimental studies has shown that other salmonids (*Oncorhynchus mykiss, O. keta, O. tshawytscha, O. kisutch, S. trutta and Salvelinus alpinus*) are susceptible for ISAV; they develop few signs of ISA, however the virus seems to be capable of propagating and they might act as asymptotic carriers (biological vectors) (Nylund et al 1994, 1995a, 1997; Nylund & Jakobsen 1995; Rolland & Nylund 1998; Devold et al 2000; Snow et al 2001a,b; Rolland & Winton 2003;Vike 2014) POMV has never been isolated from rainbow trout or been observed with clinical signs of SON, however RNA has been detected by real time RT-qPCR in a single farmed rainbow trout (Godwin et al., 2020). The susceptibility of rainbow trout and other salmonids remains to be tested.

Unlike POMV, there is no known marine reservoir for ISAV, and the natural hosts are most likely *S. salar* and *S. trutta* (Raynard et al 2001, Plarre et al 2005; Plarre 2012; Vike 2014). ISAV is known to change from low-virulent (HPR0) to high virulent variants (HPRΔ) (Nylund et al 2003, 2007, Plarre et al 2012, Christiansen et al 2017; Nylund et al 2019). The population density of *S. salar* and *S. trutta* is highest in the river, however it is too small to sustain high virulent ISA viruses (Nylund et al 2003, 2007). Therefore, the virus must consist of low virulent strains in natural populations (Nylund et al 2003, 2007; Plarre et al 2012, Vike 2014, Nylund et al 2019). Pilchards, which is thought to be the natural reservoir for POMV, have large populations in Indo-Pacific and East Pacific oceans and can sustain pathogenic strains, as seen for pilchard herpesvirus (Whittington et al., 2008). However, POMV has not been reported causing disease in this species and its susceptibility is yet to be experimentally tested (Godwin et al., 2016).

1.7 Epidemiology and transmission

Field based observation indicate horizontal transmission of POMV between individual Atlantic salmon and between pens (Godwin et al., 2016). Experimentally POMV is shown to be transmitted by direct skin to skin contact, but also to be highly infectious by indirect transmission through the water (Samsing et al., *in press*). Little is known about shedding routes or entry ports of POMV. Horizontal transmission of ISAV occurs via virus shedding from various routes, including skin, mucus, faeces, urine and blood and dead fish (Totland et al., 1996). ISAV is known to shed before observations of clinical signs and the gills to be the most probably port of entry (Aamelfot et al., 2012; Austbø et al., 2014; Mikalsen et al., 2001; Totland et al., 1996; Weli et al., 2013). However, ISAV do not remain infective for long as it

is only shown to survive for three hours in natural seawater, and up to 24 hours in sterile seawater (Vike et al., 2014). POMV seems to have a higher infectivity, even though POMV persistence in the field is not known, cultured POMV in sterile water is shown to remain infectivity for up to 14 days in a wide range of temperatures (15 - 22°C) and pH values (4. 6 – 8.7) (Morrison et al., 2013). This indicate that POMV might be transmitted longer distances than ISAV, as seen eg. salmonid alphavirus (Skjold, 2014). Despite ISAV low infectivity, dispersal of ISAV can occur over long distances as farm gear, well boats and copepods can act as mechanical vectors (Jarp & Karlsen, 1997; Murray et al., 2002; Vågsholm et al., 1994). Pilchards and marine fish are likely to act as biological vectors for POMV, as well as other vectors not yet investigated for POMV (Godwin et al., 2016).

Vertical transmission has not been shown for POMV, however low virulent HPR0 variants of ISAV is known to be vertically transmitted (Vike et al 2008; Marshall et al 2014; Nylund et al 2019). Molecular epizoology show that HPR0 is present in smolt production and in brood fish in Norway (Nylund et al 2007; Lyngstad et al 2012). In addition, ISAV from different areas are shown to be closely related and this relationship seems to reflect the origin of the eggs (Nylund et al 2007; Plarre et al. 2012, Vike et al 2008, Nylund et al 2019). This indicates that ISAV circulates in farmed salmonids via brood stock populations, and that there is little or no transmission from wild to farmed salmonids (Nylund et al 2019). Vertical transmission is therefore thought to be an important mechanism for long distance spreading of ISAV, giving rise to high prevalence and reservoir of HPR0 in farmed salmon. ISAV can further be spread locally through horizontal transmission of both HPR0 and HPRΔ variants (Nylund et al., 2019). The epidemiology and transmission routs are not necessarily similar for POMV and ISAV as they differ significantly in their reservoir and host range.

1.8 Prevention and control

Infectious pathogens, such as POMV, present a threat not only to fish health and welfare but also the environment and economies of framers. Animals in an open aquatic environment are constantly exposed to pathogens and due to high densities of fish in aquaculture can be rapidly spread (Sommerset et al., 2005). To reduce impact of diseases it is important to focus on preventing the occurrence of disease rather than treating diseased fish (Assefa & Abunna, 2018). Some key factors in the prophylaxis of disease is good fish management and hygiene in addition to limited stress (Sommerset et al., 2005). The Tasmanian industry can draw

experience from ISA as implementation of precaution to reduce horizontal spread and infection pressure has significantly reduced the number of outbreaks internationally (Håstein, 1997). This includes Norway, were the number of ISA cases increased the following years after the first official outbreak of ISA was registered (1984) (Thorud & Djupvik, 1988), reaching a peak in 1990 with over 80 outbreaks (Håstein et al., 1999). As a result, regulatory actions were introduced and the number of outbreaks decreased, and ever since 1993 the number of cases has been low, raging form 1-20 outbreaks each year (Veterinærinstituttet, 2020).

Implementing separation of generations played an important role in preventing horizontal transmission of virulent variants of ISAV. In addition to restrictions related transportation and movement of fish, mandatory health control, slaughterhouse and transport regulations, ban on sea water use during smolt production, compulsory disinfection of wastewater following slaughter, specific measures including restrictions on affected, suspected and neighbouring farms and enforced sanitary slaughtering (Håstein, 1997; Håstein et al., 1999). Simply put, ISA is regulated by strict measures and is also highly monitored, as it is an OIE listed disease and notifiable (list 2) in Norway and within the EU (European Commission, 2006). However, new research and knowledge of epizoology and transmission of ISA is still generated, and regulations should be constantly updated and be based on scientifically proven ways (Assefa & Abunna, 2018). Per example, as new evidence supports the importance of vertical transmission of ISAV, Nylund (2019) recommendations to include regulations to remove the low virulent ISAV from the brood fish, in addition to removal of the virulent variants (as the regulation are based on to this day). Similarly, a greater understanding of transmission routes, host range, reservoirs and important risk factor of SON is critical to prevent disease.

Vaccination is one of the most effective ways to prevent disease (Assefa & Abunna, 2018), however the viral vaccines are generally shown to not be as effective compered to bacterial vaccines (Crane & Hyatt, 2011; Kibenge et al., 2012; Sommerset et al., 2005). The vaccines available for ISA does not seem to give complete protection (Falk, 2014). A vaccine for POMV has been developed and was tested for the first time in 2019. However the effectiveness of the vaccine is not known as it is still being investigated as a part of a three-year POMV project (Norwood, n.d.). Diagnostic tests are also of great importance in prevention and control of disease. An accurate, rapid diagnostic test is essential to avoid

misclassification, for early detection and management of disease and to carry biosecurity programs and surveillance (Assefa & Abunna, 2018).

1.9 Diagnostic methods

Confirmation of disease due to POMV and the associated diagnostic tools are based on the same principles as ISA. Primary diagnostic methods are based on observation at the site, clinical symptoms, gross pathology and histopathology. The requirements for the diagnosis of confirmed ISA includes presence of pathological signs compatible with the disease in addition to specific detection of the causative agent ISAV (OIE, 2019). According to the World Organization for Animal Health (2019), the virion can be detected by one of the following methods, which are all based on detecting either the nucleic acids or proteins of ISAV. Virus isolation by cell culture followed by virus identification by indirect fluorescent antibody test (IFAT) or RT-PCR (conventional or quantitative). ISAV detection in histological sections by immunoassay (immunohistochemistry, IHC). Detection of ISAV in tissue preparations by PCR or IFAT. IHC and IFAT are both based on visualizing specific viral protein targeted by labelled antibodies. PCR detects POMV by amplifying nucleic acids of the virus to confirm its presence (OIE, 2019).

Diagnostic tools developed for detection of POMV include antibody-based detection by IHC and immunocytochemistry (ICC) (targeting NP, HE ans S8A proteins), situ hybridization (ISH) (targeting segment 2), and conventional RT-PCR and quantitative RT-PCR (targeting segment 5) of preferably kidney, liver and spleen (Godwin et al., 2016, 2020; Mohr et al., 2020) POMV can replicate in ASK (Atlantic salmon head kidney leukocyte) and CHSE (Chinook salmon embryo), RTG (Rainbow trout gonad) cell lines. CPE for POMV includes ‘rounding’ of infected cells and partial detachment from the monolayer (Godwin et al., 2016; Mohr et al., 2020)

1.10 What makes an accurate diagnostic tool?

Diagnostic test accuracy refers to the ability of the test to distinguish diseased individuals from healthy individuals (healthy individual can be infected by a virus, ex ISAV HPR0), or a diagnostic test can distinguish between positive specimens (infected by a specific agent) and negative specimens (not infected) (Lalkhen & McCluskey, 2008; Maclachlan & Dubovi,

2010). The accuracy of a test depends on the specificity of the approach used (assay), i.e. the test should only give positive result for one specific agent (pathogen). A 100% specific test will never give false positives (unless contamination occur). The sensitive of a test is the ability to detect all positive individuals, i.e. a 100 % sensitive test will never give false negatives. However, there are no test that have a sensitivity of 100 % which mean that there is always a risk of false negatives (Aslam et al., 2016; Parikh et al. 2008). Generally, virus isolation in cell culture is considered as the "*gold standard*" tests for viruses, since the method shows the presence of infectious virions that can be identified. However, use of cell cultures has a high cost (time consuming) and lack of available cell cultures for fish viruses limits the use of this approach. The most used alternative for detection of virus infections is RT-qPCR which a rapid, sensitive and highly specific method for identification of viral genes (Maclachlan & Dubovi, 2010). This method cannot be used for quantification of virions (within an infected host the are several different RNAs that will be detected by the assays, ie. not only genomic RNA from virions), but can, in most cases, give a good indication of production of virions within the hosts. A correct diagnosis will have to include observation of pathology (behavioural changes, gross pathology and histopathology) combined with detection of specific virions (or viral RNA or viral proteins) known to cause the pathology (OIE, 2019).

1.11 Origin of the project

This master project initiated from a larger POMV project investigating virus transmission and infection dynamics. This project raised from recent gene expression data conducted by next generation sequencing in ASK-cells, kidney and liver showing higher expression of other segments than segment 5 of POMV at an early infection state (Samsing et al. *in press*). This finding indicated that other segments of the viral genome could be better candidates for early detection of the disease than the currently used RT-qPCR assay targeting segment 5. For early detection, understanding of the virus distribution in the tissue is also desirable, such as knowledge of initial site of infection for efficient sampling. Sub-clinical fish were of interest to test as the fish is infected but without clinical signs and can be easily overlooked. It would be desirable to be able detect this infection phase and thereby eliminate infected animals and implement control strategies at an early stage.

1.12 Thesis Aims

Pilchard orthomyxovirus (POMV) is a virus of major concern to the salmon farming industry in Tasmania. The virus was first isolated from Atlantic salmon in Tasmania in 2006, and since 2012 it has been increasingly associated with stock losses and severe disease outbreaks. Early detection of POMV could significantly improve management of the disease, reduce losses and ultimately improve animal welfare. The main aim of this thesis is to develop diagnostic tools to improve subclinical detection of POMV. The specific aims of this thesis are:

1. To investigate viral tissue tropism during a time course infection in experimentally infected Atlantic salmon, *Salmo salar.*
   - Conducting real time RT-qPCR using the current segment 5 assay, immunohistochemistry and virus titration on tissues collected at defined time points following the challenge (potential subclinical animals).

2. To develop and evaluate new targets for the detection of POMV using molecular tools
   - Design, validation of reaction (efficiency and analytical sensitivity) and optimization of the two new Taqman real time RT-qPCR assays.
   - Analytical specificity with respect to other Tasmanian viruses, including Tasmanian Aquabirnavirus (TABV), and Tasmanian salmon Aquareovirus (TSRV), in addition to the exotic virus ISAV.
   - Conduct diagnostic sensitivity and specificity testing of the two newly developed Taqman real time RT-qPCR assays and compare them to the current segment 5 assay on subclinical, moribund and naïve fish.

2. Material and methods

2. 2.1 Experimental set up

The cohabitation infection challenge was conducted in 2018 in Tasmania, Australia. Planning or executing of the experiment is for that reason not part of this master thesis. The experiment is described to provide information of the samples that were analysed in the master's project.

2.1.1 Fish

Female diploid Atlantic salmon (*Salmo salar*) smolts of mean weight 116 g were obtained from a commercial tank cohort at the Florentine hatchery (Salmon Enterprises of Tasmania) on September 2018. The fish had previously been treated against yersiniosis (*Yersinia ruckeri* serotype O1b biotypes 1 and 2) by dip-vaccination with Yersinivac-B® (Tréidlia Biovet, Sydney) as fry at 2.5g and 8g in spring 2017. Subsequently, by intraperitoneally (IP vaccination with Yersinivac-B-injectable at parr stage in July 2018. The cohort were exposed to L10:D14 (light:dark) photoperiod followed by seven weeks of L24:D0 to trigger smoltification. Prior to transfer, the fish was independently tested for disease by bacterial culture of kidney on blood agar and histopathology of major organs by the Animal Health Laboratory of the Department of Primary Industries Water & Enviroment (DPIPWE).
The smolt were transported to DPIPWE Biosecure Fish Facility (Prospect, Tasmania) in a 1 m^3 fish transport tank with automated oxygenation (95 – 105% oxygen saturation) in hatchery water (7.5°C). Over two weeks, the fish were maintained in a recirculatory system (RAS) composed of 12 individual 1000 L tanks of freshwater (11 °C ± 1). In the second week, the salinity and temperature were gradually increased, resulting in full marine conditions with a salinity of 32 ppt and temperature of 15 °C ± 1. Sea water was obtained from the Tamar estuary in the north of Tasmania and was chlorinated (≥5 ppm for 12-24 h) and dechlorinated prior to use.

According to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8th edition, 2013), all animal procedures were approved by the DPIPWE Animal Ethics Committee (AEC) (AEC no. 3/2018-19).

2.1.2 Preparation of inoculum

Chinook salmon embryo cell line (CHSE-214) were grown at 20 °C in 175 cm^2 flasks with Eagles minimal essential medium (EMEM) containing L-glutamine (Sigma-Aldrich) and supplemented with 10% (v/v) Australian foetal bovine serum (Invitrogen, Life Technologies). The POMV isolate TCFV 0067-0004 (virus passage number four) was used as inoculum and propagated in 90-100% confluent cell monolayers. The cultures were observed daily by inverted light microscopy for development of cytopathic effects (CPE). When 100% of the monolayer had CPE present, the supernatants were transferred into a single flask. The

supernatants were clarified by centrifugation at 4,500 x g for 15 min at 4°C and passed through a 0.2 µm filter to exclude cell debris. Subsequently aliquoted and stored at -80°C. Virus titres were calculated according to the 50% end-point method of Reed and Muench (1938) and expressed as tissue culture infective dose 50% per ml ($TCID_{50/mL}$).

2.1.3 Cohabitation Infection Challenge

The cohabitation infection challenge was designed to test direct and indirect transmission of POMV between Atlantic salmon in sea water. The experiment involved a paired tank set up, consisting of one cohabitation tank with fish injected with POMV (*trojans*) and naïve fish (*co-habitants*), and one indirect cohabitation tank with naïve fish (*indirects*). The two tanks (2 x 1000 L) were connected by an unfiltered pipe circulating seawater between them (26 $1min^{-1}$) exposing the indirects to POMV through the water (Fig. 6). The paired tanks were run in four replicates where three of the tanks were sampled throughout the experiment. The fourth replicate was used to quantify virus-associated morbidity/mortality. The water temperature was kept at 15 °C ± 1.

On day zero of the experiment, the trojans were anesthetized with 20 ppm AQUI-S™ and IP-injected with 200 µL of cell culture supernatant containing POMV at titre of $1x10^{8.8}$ $TCID_{50}$/mL CHSE-214 using 1 mL graduated syringes and 6 mm x 22 G needles (Unimed, Lausanne, Switzerland). To distinguish the trojans from the non-injected fish, the adipose fin of the trojans was clipped. The experiment was conducted with the cohabitation tanks stocked with 22 trojans and 32 co-habitants, and the indirect cohabitation tank had 32 indirects for each individual tank.

The tanks were monitored three times a day during the trial, which was finalized 20 days post injection. Moribund fish, fish showing clear signs of POMV, were removed immediately, euthanized with mg L^{-1} of AQUI-S™ and necropsied. Clinical sign of POMV is described in Godwin (2020) and includes swimming near the surface of the tank, lack of response to stimuli and dark coloration of the skin. The trial was terminated when morbidity in the cohab and indirect groups reached a consistent plateau for at least three days.

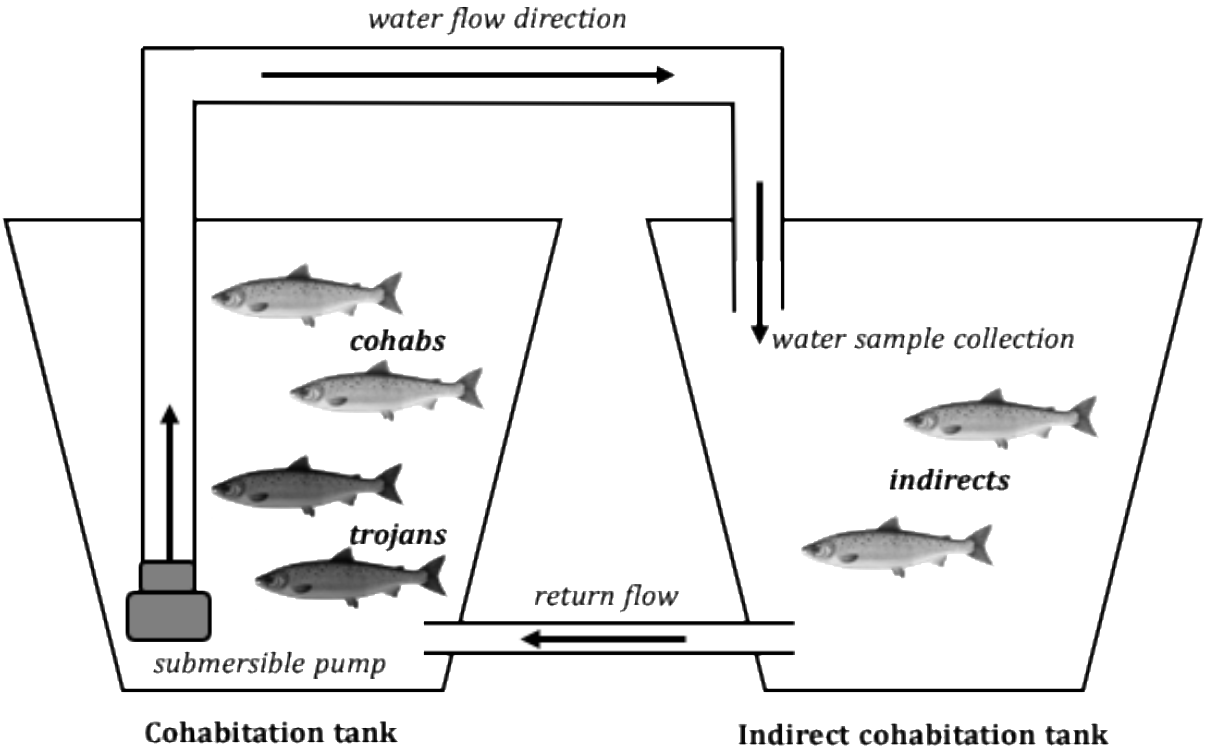


Figure 6 Schematic representation of the two challenge models (cohabitation and indirect cohabitation) used in this experiment to examine the transmission of Pilchard orthomyxovirus (POMV) in seawater. Paired tanks (2 × 1000 L tanks) were coupled together so that seawater circulated through both via a piping system, but fish in each tank were prevented from coming into direct contact. Dark grey fish in the cohabitation tank represent fish that received an intraperitoneal (IP) injection with POMV (*trojans*) at a titre of 1 x $10^{8.8}$ $TCID_{50/mL.}$ Lighter grey fish were exposed to the virus via direct cohabitation (cohabitation tank) or via seawater (indirect cohabitation) coming from the cohabitation tank. Cohabitation tanks were stocked with 22 *trojans* and 32 *cohabitants*, and indirect cohabiration tanks with 32 *indirects*. Adapted from Samsing et al. (*in press*).

2.1.4 Sample Collection

On day zero of the experiment, nine naive fish were sampled from a holding tank before allocation of fish into the four paired tanks. Observed moribund fish were removed and sampled throughout the experiment. During the cohabitation infection challenge, three fish were sampled from each tank and treatment (cohabitation and indirect exposure tanks) on days 1, 2, 4, 8, 12- and 19-days post-injection of trojan fish. The fish sampled were cohabs and indirects which had absence of clinical signs of POMV. These fish were assumed to be subclinical infected individuals, however, it is also plausible that these fish are in the early stages of SON and thereby pre-clinical and would eventually develop clinical signs. To

confirm the actually state of the animal, a non-lethal sampling method is required to individually monitor the same fish over time, which was out of the scope for this study.

Samples for quantitative Polymerase Chain Reaction (RT-qPCR) were collected in RNA*later*® and stored at 4°C overnight and placed in -80°C until further use. The samples collected and analyzed were gill arch, spleen, liver, head-kidney and heart. The same tissues, in addition to mid-gut, were collected in 10% buffered formalin for analysis by immunohistochemistry. For virus isolation, samples of spleen, liver and head-kidney were collected in bead homogeniser tubes (Lysing Matrix E 2 mL tubes, MP Biomedicals™ Tubes) and stored at -80°C.

In attempt to answer the aims of this thesis sub-clinical fish samples were used and analyzed for examination of tissue tropism and for testing of the POMV RT-qPCR assay. We did not differentiate between cohabs and indirects but treated all of the samples "equal" by simply grouping them as sub-clinical fish. Only a few samples of naïve and moribund fish were supplied to this study. Moribund fish were used as positive controls for RT-qPCR and virus titration, and a few samples of moribund and naïve fish were also supplied for testing of the new RT-qPCR assays.

2.1.5 RNA-extraction

To isolate RNA and remove genomic DNA contamination, RNeasy® Plus Mini kit (QIAGEN, Venlo, Netherlands) was used according to the manufacturer's protocol: "Purification of Total RNA from Animal Tissues". All centrifugation steps were performed at 14000 rpm with Eppendorf centrifuge 5417 R and all work involving β-Mercapoethanol was performed in a fume hood.

Tissue samples stored in RNA*later*® were dissected to specimens of ~ 20 mg, minced with a scalpel, and placed into a Lysing Matrix E 2ml columns (MP Biomedicals, USA). RLT buffer / β-Mercapoethanol (600 µL) was added and the tissue was further disturbed by the TissueLyser II (QIAGEN, Venlo, Netherlands) to a uniform homogenous solution by 3 mins at 20 hz. The lysate was centrifuged for 3 min and the supernatant was removed by pipetting (~ 600 µL). This was transferred to a gDNA eliminator spin column (provided by the kit) placed in a 2ml collection tube, and centrifuged for 30 s. One volume (~ 600 µL) of 70%

ethanol (50% ethanol for liver samples) was added to the flow-through and mixed by pipetting. A portion of the lysate-alcohol mix (~ 600 µL) was immediately transferred to a Rneasy spin column placed in a 2 ml collection tube and centrifuged for 15 s and the flow-though was discarded. This was repeated with the remaining lysate-alcohol mix (~600 µL).

The Rneasy spin column was then washed thought several steps. Following the final wash, the Rneasy was transferred into a new 2 ml collection tube and centrifuged for 1 minute to remove residual ethanol and wash buffer. Then after RNA was eluted, the Rneasy spin column was placed into a 1.5 ml Eppendorf tube, and 40 µL of Rnase free water was added directly to the spin column membrane. After a 1 min incubation at room temperature samples were centrifuged for 1 min. An aliquot of the eluted RNA was taken for analysis and the remaining sample was stored at -80 until further use.

A NanoDrop 8000 Spectrophotometer (Thermo Scientific, Waltham, Massachusetts USA) was used to measure total RNA concentrations and the A260/A280 and A260/A230 ratios for purity. RNA was quantified in order to normalize the input value for RT-qPCR reaction to a concentration of 10 ng/µL then stored at -20°C. Values above 2.0 for A260/280 and 2.2 for A260/230 is recognized as "pure" (Desjardins & Conklin, 2010) and were further analyzed.

2.1.6 Real time RT-qPCR

All RT-qPCR analyses were run in a QuantStudio™ 5 Real-Time PCR System (ThermoFisher™) according to standard AgPath-ID one-step RT-PCR kit (ThermoFisher™) setup, which is 45°C for 10 minutes, 95◦°C for 10 minutes and then 40 cycles with 95°C for 15 seconds and 60°C for 45 seconds. The RT-qPCR reactions were run as (1) standard or (2) reduced reaction volume.

(1) A standard 25 µL reaction included (per reaction); 2 µL template (10 ng/µl) RNA, 12.5 µl 2x RT-PCR buffer, 1 µL 25x RT-PCR enzyme mix, primers/probe.

(2) A reduced 10 µl reaction included (per reaction); 1 µL template (10 ng/µl) RNA, 5 µl 2x RT-PCR buffer, 0.4 µl 25x RT-PCR enzyme mix, primers/probe.

The primer and probe concentrations varied depending on the assay used (Table 2).

Table 2. Real-time PCR primers and Taqman probes to detect POMV segment 5, segment 7, segment 8 and ELF1α

Gene	Primer	Sequence (5´-3´)	Reaction conc. (nM)	Annealing temp (°C)	Amplicon length (bp)	Reference
POMV segment 5	POMV F	ATCAGAAGGGACGGTGGAAG	900	60	112	Mohr et al., 2020
	POMV R	CGGTCTCGCAACTCTTGATTG	900			
	Taqman probe	FAM-TCAAGCCCAAGGAACCGCAAAAGC-TAMRA	250			
POMV segment 7	POMV F	CACGAGATTGCCGACGACTT	200	60	132	This study
	POMV R	CTCAGCAAGGATCGCCACAA	900			
	Taqman probe	FAM.AGCCGCTCAGATTGACGGACCTG-TAMRA	250			
POMV segment 8	POMV F	GAACATCATCTCCACGACTCA	200	60	80	This study
	POMV R	CACCACTCTTAACTCCCTTGG	900			
	Taqman probe	FAM-TGCGGCAGATCGAAACCATCCT-TAMRA	120			
ELF1α	ELF1α F	CCCCTCCAGGACGTTTACAAA	900	60	57	Snow et al. 2006
	ELF1α R	CACACGGCCCACAGGTACA	900			
	Taqman probe	FAM-ATCGGTGGTATTGGAAC-TAMRA	200			

A small experiment was conducted to compare full and reduced Agpath reactions and resulted in that the two yielded comparable Ct-values (Appendix 1). The reasoning for reducing the reaction was to be able to test more samples and was only used for samples tested to investigate "Diagnostic sensitivity of different POMV assays"**.**

The endogenous control translation elongation factor 1 alpha (ELF1α) (Snow et al., 2006) was run against each sample as a reference gene due to its stable expression in Atlantic salmon (Ingerslev et al., 2006). The reference gene was included to normalize the Ct-values variation between samples as a result of varying quantity and quality of tissue and RNA extraction. Values obtained from the qRT-PCR analysis by the segment 5, 7 and 8 assays were set up against values obtained for the ELF1alpha endogenous control in each case. These calculations were performed according to the delta-delta Ct method, also known as the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) in Excel.

Samples were run in duplicates for the reference gene (ELF1α) and triplicates for the various POMV segments and had a standard deviation $\leq$0.5 (Agilent technologies, 2012). Samples were determined as POMV positive detections if a Ct value was detected above the threshold in at least two of the three technical replicates, and then reported as an average Ct-value. For each of the given assay (i.e. same primer and probe set) the same threshold value settings were used for every run to improve the precision and make the data more comparable from plate to plate and instrument to instrument, and the baseline was set to auto (Applied Biosystems, 2002). Non-template controls (NTC) and a positive control were included in every run to ensure there was no contamination and right mastermix setup was followed, respectively. The positive control was a head kidney from a moribund fish tested very positive (Ct <25) for POMV by the segment 5 assay by RT-qPCR.

3. 2.2 Viral tissue tropism

Three methods were conducted to investigate the viral tropism of POMV in subclinical Atlantic salmon over a time course infection on tissues collected 1, 2, 4, 8, 12- and 19-days post infection from initiation of the cohabitation. The methods include qRT-PCR, viral titration and immunohistochemistry and are thus all based on fundamentally different strategies for detecting viral components.

2.2.1 Real time RT-PCR (PT-RT-qPCR) of Segment 5

To investigate viral tropism the currant assay for POMV detection, the segment 5 RT-qPCR assay (Mohr et al., 2020), was performed on gill arch, liver, head-kidney, heart and spleen for six individuals per time point during the cohabitation challenge. Both assays, POMV segment 5 and ELF1α, were performed in a 25 µl with primer and probe concentrations specific for each assay (Table 2.).

2.2.1 Virus titration

Virus titration was conducted to investigate the viral tropism of (viable) POMV in Atlantic salmon over a time course infection, and was performed on liver, kidney and spleen for three individual per time point during the cohabitation challenge.

Chinook salmon embryo (CHSE-214) was grown in Eagle's Minimum Essential Medium containing L-glutamine (EMEM Sigma-Aldrich, Castle Hills, NSW) and supplemented with 10% foetal bovine serum (FBS, Victoria) in a roller bottle. The bottle was rinsed with 40 ml TrypLE Select (Gibco, Thermo Scientific, Waltham, Massachusetts US), decanted and fresh 40 ml TrypLE was added and rolled on a roller platform at 18 rpm for 30 min. The detached cells and supernatant were collected into a 50 ml Falcon tube. 30 ml of Media (10% EMEM) was added to the roller bottle, rinsed and supernatant collected. The cells were centrifugated at 1000 rpm for 5 min and the supernatant decanted. Pooled cells were resuspend in a total of 10 ml of 10% EMEM and mixed by > 20 pipette motions. Cells were counted with countess (Thermo Scientific, Waltham, Massachusetts USA), diluted to $3.5x10^6$ cells and seeded in a 96 well plate by adding 150 µL of cell per well (1 plate per tissue sample) and incubated at 20 ºC overnight with 2% CO2.

Salmon tissues stored at -80ºC were collected, dissected and weight of tissue recorded minus an average weight of bead homogenizer tubes. Tissues thawed on ice were homogenized in homogenizer tubes with 500 µl of viral transport medium (VTM) (HBSS+ 2% Foetal bovine serum (Serana, Australia) + 2% Pen/Strep (Gibco, Australia) using a Precellys Evolution homogenizer (Bertin Instruments, Thermo Fisher Scientific, Melbourne) under cold conditions between 2-10 ºC at 8000 rpm for 4 x 15 seconds pulses with a 30 second pause between each puls.

Thereafter each tube was centrifuged to pellet tissue fragments and the supernatant collected. The dilution of tissue arising due to homogenization was calculated based on the recorded weight of the tissue for each sample. The supernatant was then diluted in VTM according to the weight of the original sample to adjust the final dilution to Xg tissue/ Yml VTM. These samples were designated 10^0. The samples were further diluted by 10-fold serial dilution in 2% FBS in EMEM. Once diluted, 50 µL of the samples were transferred to a 96-well plate containing 150 µL of $3.4x10^6$ CHSE-214 cells in 10% FBS EMEM in each well, which were prepare the day before use. The plates were then incubated for 21 days and the wells were visually inspected for signs of characteristic POMV-like CPE (Mohr et al., 2020). The $TCID_{50}$ was calculated according to the Reed and Muench $TCID_{50}$ calculation method (Reed & Muench, 1938)

2.2.3 Immunohistochemistry (IHC)

Immunohistochemistry was conducted on six individual fish per time point for gill arch, liver, head-kidney, heart, mid-gut and spleen samples. IHC was processed using a polyclonal anti-POMV antibody (Morrison et al., in press) as described in Samsing et al. (*in press*). In brief, antigen staining in each tissue sample was assessed using a light microscope and scored at ×200 as either undetectable (NEG), minimal (+, between one and five positive foci per field of view), moderate (++, between six and ten positive foci per field of view) or abundant (+++, more than ten positive foci per field of view). Immunohistochemistry and subsequent analyses were performed at the Australian Animal Health Laboratory (AAHL) in Geelong, Australia, by Chloe English (PhD candidate).

4. 2.3 Development of new diagnostic PCR tests

As discussed ISAV is the closest relative to POMV. The recognised method for detecting ISAV is with RT-qPCR assays for segments 7 and 8 (M Snow et al., 2006) and are commonly used during disease outbreak and in apparently healthy fish (OIE, 2019).

Recent gene expression data indicate that other segments of the viral genome of POMV could be better candidates for early detection of the disease. To improve sub-clinical detection of POMV other potential targets of the POMV genome were explored. The target choice was based on evidence of high expression of the two segments early in the infection (6- and 24-hours post infection) by next generation sequencing data in ASK-cells, kidney and liver

(Samsing et al, *in press*). Subsequently, two new Taqman RT-qPCR assays targeting segment 7 (ORF 1) and 8 of POMV were designed with the hope of developing a more sensitive and specific screening assay.

2.3.1 Selection of suitable primers and probe

In order to design the primers and probe for each assay, alignments were conducted in Molecular Evolutionary Genetics Analysis (MEGA, version 7). To ensure detection of all documented variants of POMV, conserved gene regions were identified by alignments of the entire ORF of all 11 available POMV isolates of the respective segment (Mohr *et al*., 2020). To ensure specificity, identical regions of POMV were further tested to be different to ISAV by aligning them with the representative ORF of European (Glesvaer /2/90) and North American (CCBB). The ISAV sequences were imported from the National Center for Biotechnology (NCBI) website. Regions that fulfilled these criteria were inserted into software with different algorithms for primers and probes design. Segment 7 and 8 were designed using genscript (Genscript, n.d.) and Primer 3 (Untergasser et al., 2012) respectively following the guidelines for primer and probe design summarized in Table 3 and 4. The primer- and probe binding sites were identical among all of the POMV isolates and contained at least 10 mismatches with aligned ISAV sequences as per design of segment 5 (Mohr et al, 2020) (Appendix 2).

The theoretical specificity of primers and probes was assessed using NCBI nucleotide Basic Local Alignment Search Tool to identify potential non-target amplification. The primer pairs were ordered from Integrated DNA Technologies (IDT) (Table 2).

Table 3. Summarized guidelines for primer (forward and reverse) design for developing segment 7 and 8 Taqman assays. Adapted from English (2019) and Life Technologies Corporation (2014)

Rule	Segment 8	Segment 7
Used region of 'clean/complete' sequence	✓	✓
Optimal length for 20 bases	✓	✓
Tm 57 – 60°C	✓	✓
NCBI check	✓	✓
%GC 30-80%	✓	✓
3' end last 5 nucleotides have no more than 4 G + C	✓	✓
No overlap between probe and primer	✓	✓
Avoid repeating oligonucleotides (< 4 Gs)	✓	✓
Avoid secondary structures (hairpins)	✓	✓
Tm difference between primers 2°C	✓	✓
Tm difference between primer/probe ~10°C	✓	✓
Amplicon length 50 - 150 (shorter is best)	✓	✓
Check primers 100% match consensus sequence	✓	✓

Table 4. Summarized guidelines for probe design for developing segment 7 and 8 Taqman assays. Adapted from English (2019) and Life Technologies Corporation (2014)

Rule	Segment 8	Segment 7
Used region of 'clean/complete' sequence	✓	✓
Must have at least 3 different base pairs	✓	✓
Length 13-25 bases	✓	✓
NCBI checks	✓	✓
Tm is 65 – 67°C	✓	✓
%GC 30-80%	✓	✓
5' end cannot be a G residue	✓	✓
Avoid repeating oligonucleotides (< 4 Gs)	✓	✓
Avoid 6 consecutive A residues	✓	✓
Avoid CC in middle of the probe	✓	✓
Avoid 5' - …GGG-MGB-3' or 5' - …GGAG-MGB-3'	✓	✓
Check probe seq. 100% match consensus sequence	✓	✓

2.3.2 Identification of amplicons

Before ordering the probes, the amplicon derived from traditional end-point PCR using each of the newly developed primer sets for segment 7 and 8 of POMV was sequenced to confirm the correct target was being amplified. RNA extracted from a strong positive POMV tissue, analyzed by the use of the segment 5 RT-PCR assay, was transcribed into cDNA by Superscript® IV Reverse Transcriptase with Oligo d(T) primers according to the protocol (Thermo Fisher Scientific, Waltham, USA). The cDNA (1 µl) was used as template in each standard 50 µl reaction mixture of the Platinum Taq DNA polymerase High Fidelity, containing (per reaction) 5 µl 10X High Fidelity PCR Buffer, 2 µl $MgSO_4$ (50 mM), 1 µl dNTP Mix (10 mM), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 0.2 Platinum® Taq DNA Ploymerase High Fidelity (5U/µl), and 38.8 µl nuclease-free water (Thermo Fisher Scientific, Waltham, USA). The PCR was performed at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 15 seconds, annealing at 55 °C for 30 seconds, elongation at 68 °C for 30 seconds in an Eppendorf Mastercycler EP Gradient S (Hamburg, Germany). PCR-products of desired size were confirmed by performing a 3% agarose gel electrophoresis using the 50 bp DNA Ladder (New England Biolabs, Massachusetts, USA) as a reference ladder. All PCR products were documented with the SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific, Waltham, USA) (Fig. 17, Appendix 3).

The PCR-products were purified by QIAquick® PCR purification Kit (QIAGEN, Venlo, Netherlands) following the manufacturer's instructions. The purified DNA was ligated into a pGEM-T easy vector (Promega, Madison, USA) (Fig 18., Appendix 3) then transformed using One Shot® TOP10 chemically competent *E. coli*. (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's protocol The transformed cells were plated onto LB agar plates containing ampicillin (100mg/L) and incubated over night at 37 °C. Successful ligation and transformation was confirmed by PCR of multiple (2-4) colonies using M13 vector specific primers, F: 5'd[CCCAGTCACGACGTTGTAAAACG]3´, R: 5´d[AGCGGATAACAATTTCACACAGG]3´. The colonies were added into separate PCR-tubes containing 5.35 µl water, thereafter 6.75 µl GoTaq® Green Master Mix, 2X, 0.2 forward primer (10 µM), 0.2 reverse primer (10 µM) was added (GoTaq® Green Master Mix, Promega, Madison, USA). Finale volume for the mastermix was 12.5 µl. The PCR protocol was 95 °C for 10 min followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds , elongation at 72 °C for 1 min, followed by 72 °C for 3

min in an Eppendorf Mastercycler EP Gradient S (Hamburg, Germany). All PCR products of desired size was confirmed performing a 2% agarose gel electrophoresis using Tridye 2-Log DNA Ladder (New England Biolabs, Massachusetts, USA) as a reference ladder. All PCR products were documented with the SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific, Waltham, USA) (Fig. 19, Appendix 3).

Colonies with inserts were grown in LB broth overnight at 37 °C. Corresponding glycerol (20%) stocks were prepared for archiving and plasmid was purified using PureYield™ Plasmid Miniprep System (Promega Madison, USA) following the "Alternative Protocol for Larger Culture Volumes" and the concentration was measured by Qubit dsDNA BR assay kit (Thermo Fisher Scientific,Waltham, USA). The purified plasmids together with M13 forward primer were sent to Ramaciotti Centre for Genomics (UNSW Sydney) for Sanger sequencing in one direction. To confirm the right sequence was being amplified, the sequencing results were aligned with the corresponding desired amplicon for each segment in MEGA (Fig. 21, Appendix 3).

Prior to sequencing, the insert direction in the vector was tested by the use of two different mastermixes for each segment: 1) M13 reverse primer and segment specific forward primer and 2) M13 reverse primer and segment specific forward primer. The PCR-reaction followed GoTaq® Green Master Mix (Promega, Madison, USA) and the PCR-products were visualized by gel electrophoresis as described in the above section. The orientation of the insert was determined by the presence of a band for *either* mastermix 1 or 2 by gel electrophoresis for each colony (Fig. 20, Appendix 3).

2.3.3 Optimization of primer and probe concentrations

To obtain a high analytical sensitivity for the newly developed assays the optimum probe and primer concentrations for a single-plex assay was determined by a series of experiments. The concentrations tested were;

1) High; primers: 900 nM, probe: 250 nM - concentrations used by the segment 5 assay (Mohr et al, 2020)
2) Mid; primers: 400 nM, probe: 120 nM - recommended Agpath concentrations (*AgPath-ID One-Step RT-PCR Reagents*, n.d.)
3) Low; primer: 200 nM and probe: 60 nM

All reactions were performed in a 25 µl reaction with RNA template from a strong positive POMV tissue, analyzed by the segment 5 RT-qPCR assay.

The primer concentrations (forward and reverse) were first evaluated. A matrix of reactions was created where a range of concentrations for each primer were tested against different concentrations of the partner primer (Table 5). The probe concentration was held consistent at 250 nM for all of the initial reactions. The primer concentration combination with the lowest average Ct yield was further tested against a range of probe concentrations (Table 6). The optimal assay was identified as the combination of TaqMan® probe and forward- and reverse-primers yielding the lowest average Ct (Appendix 4).

Table 5. Optimization of forward and reverse primer concentration against AgPath-ID™ One-step kit for segment 7 and 8.

Forward/reverse	F primer (200nM)	F primer (400nM)	F primer (900nM)
R primer (200nM)	200/200	400/200	900/200
R primer (400nM)	200/400	400/400	900/400
R primer (900nM)	200/900	400/900	900/900

Table 6. Optimization of probe concentrations against AgPath-ID ™ One-step Kit.

Probe concentrations (nM)			
60	120	180	250

As per the segment 5 RT-qPCR assay, the threshold was set to 0.1 for both Segment 7 and 8 assays. This was a region where exponential amplification was seen across all amplification plots and depicted in the log view of the amplification plots as linear (Applied Biosystems, 2002). Subsequent assays were run with the optimized concentrations and set threshold settings.

2.3.4 Reaction efficiency and LOD of the segment 7 and 8 assays

To determine analytical sensitivity, an efficiency test was performed to determine segment 7 and 8 assays' ability to detect their respective target template. This was done by using amplicon specific plasmid DNA for each of the segments. The copy number corresponding to each sample of plasmid DNA was determined by submitting the concentration (measured by a Qubit TM dsDNA HS Assay Kit, Thermo Fisher Scientific,Waltham, USA), together with the

length of the plasmid (amplicon insert length added to the vector length) to the online DNA/RNA copy number calculator Endmemo (no date). The plasmid DNAs were then diluted 10-fold and a standard curve was conducted from 10^7 to 10 copy/µL water for each segment in a 25 µL Agpath reaction mixture with the optimized primer and probe concentrations. Each dilution point of the standard curve was run in triplicates, and the mean Ct-value was plotted against the dilution series in Microsoft Excel where slope and regression for the graphs were calculated. The efficiency was then calculated using the formula $E= 10^{(-1/slope)}*100$ and the linearity was demonstrated with the coefficient of determination (R^2).

Following English et al. (2019), the 10-fold dilution series was then used to determine the limit of detection (LOD) for each assay. For this purpose, the lowest dilution that provided a Ct value in all 3 replicates underwent a 2-fold dilution and was tested in quadruplicate. The mean of the lowest dilution of the 2-fold dilution that provided a Ct value in all replicates was determined as the LOD.

2.4 Diagnostic sensitivity of different POMV assays

To assess the diagnostic sensitivity of the newly developed assays, RNA extracted from sub-clinical and moribund fish were tested using the three assays targeting different segments of the viral genome: segment 5, 7 and 8. The sub-clinical samples tested were head kidneys found positive to either virus titration, immunohistochemistry or/and the segment 5 RT-qPCR assay for the time course experiment. Samples with a Ct value only detected in one of the three technical replicates by the segment 5 RT-qPCR assay through the time course experiment were considered a suspect of virus presence and was therefore also tested. The head kidneys were analyzed as its one of the tissues used in current diagnostic testing of POMV (RT-qPCR targeting segment 5), its one of the suitable tissues for virological examination according to OIE (2019) (both sub-clinical and clinical affected fish) and it showed the earliest detection of POMV according to the time course experiment data. Each assay was performed in 10 µL Agpath reactions (AgPath-ID one-step RT-PCR kit, Life technologies) with primer and probe concentrations specific for each assay as previously described (Table 2). The data generated was further normalized against ELF1α.

None of the existing reference methods for detection of POMV is considered diagnostically perfect (i.e. gold standard). The diagnostic sensitivity for each assay was therefore estimated

against a ‘diseased’ class, which included animals that were positive to either of the existing reference methods, namely RT-qPCR segment 5, virus titration, or IHC.

2.5 Diagnostic specificity

Diagnostic specificity (DSp) was investigated for the three assays targeting segment 5, 7 and 8 of POMV by testing samples of naïve fish (pre-challenge negative controls, n=5).

2.6 Statistics

The statistical analysis and plots were conducted and rendered in RStudio (Version 1.1.419 – RStudio, Inc. http://www.rstudio.com/), using R (Version 3.5.1 – R Core Team, http://www.Rproject.org/). The tissue tropism data, RT-qPCR (segment 5 assay as relative quantification against ELF1α), virus titration ($TCID_{50}$) and antigen staining scores from immunohistochemistry, was analyzed by Linear mixed-effects models (LMMs). Fixed effects in the LMMs included all terms (time-points, tissue and exposure method) and appropriate interactions, using fish unique IDs nested within tanks as a random factor. To examine the effect of tissue within each day individual 2-way ANOVA was used for each, with day (dpi) and exposure method as factors in the model. Tukey post-hoc test was used to examine pair ways differences in tissues. To investigate the sensitivity of the POMV assays, targeting segment 5, 7 and 8, mcnemar test was used to generate 2 × 2 contingency tables. Significant differences between the assays’ ability to determine fish as diseased or non-disease were examined by paired ways comparisons. Ct-values as relative quantification against ELF1α ($\log 2^{-\Delta\Delta Ct}$) were fit to LMMs with Fish IDs nested within state as a random factor, and assay as a fixed effect. Significant differences were compared using post-hoc Tukey’s test. Assumptions of normality and homogeneity of variance were evaluated with plots of model residuals. For all statistical analyses, the significant *p-value* was set at 0.05 or less.

3. Results

The cohabitation infection challenge was conducted in 2018, and for that reason I did not contribute in planning or executing the experiment but has analyzed samples obtained from the experiment.

3.1 POMV pathology and morbidity

The cohabitation challenge of Atlantic salmon post-smolts composed of a paired tank set up of a cohabitation tank with fish injected with POMV (*trojans*) together with naïve fish (*cohabs*) and indirect cohabitation tank with naïve fish (*indirects*) exposed to the virus through a waterpipe from a cohabitation tank (Fig. 1). The experiment resulted in morbidities and mortalities throughout the experimental period. Mortalities of trojans were observed four days post injection (dpi) and increased until 12 dpi when it reached 57.1% morbidity. Cohab morbidity started 6 dpi and peaked at 18 dpi with 51.5% morbidity. Moribund fish were first observed 7 dpi in the indirect cohabitation group, and the number slowly increased to a total of 15.6% morbidity at 19 dpi (Fig. 7).

Clinical signs in moribund fish were compatible with the disease caused by POMV (Godwin et al., 2020). Initial signs of disease included a reduction in feed intake, yellow/white casts and loose scales in the water column. Fish were swimming near the surface of the tank, lethargic and showed reduced responsiveness. Fish had dark pigmentation of the skin, red coloration and haemorrhages around the vent and ventral areas of the body, and exophthalmia was present in some individuals. Other observed signs found at the necropsy included splenomegaly, presence of mucus in the stomach and intestines, and vascular changes (petechial haemorrhaging of visceral fat and internal organs) (Fig. 8).

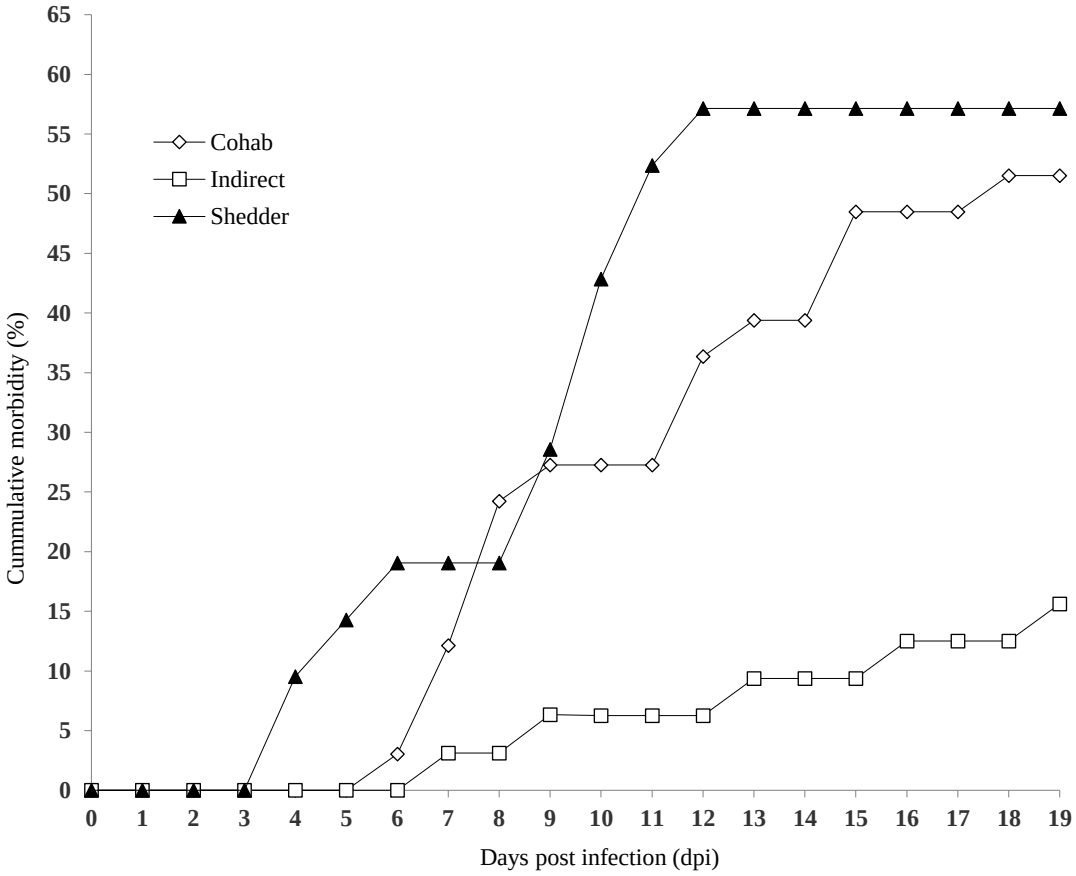


Figure 7. Cumulative morbidity of fish challenged with Pilchard orthomyxovirus (POMV) using three different exposure models: intraperitoneal (IP)-injection (*trojans*), cohabitation (*cohabs*) and indirect-cohabitation (*indirects*). Adapted from Samsing (*in press*).

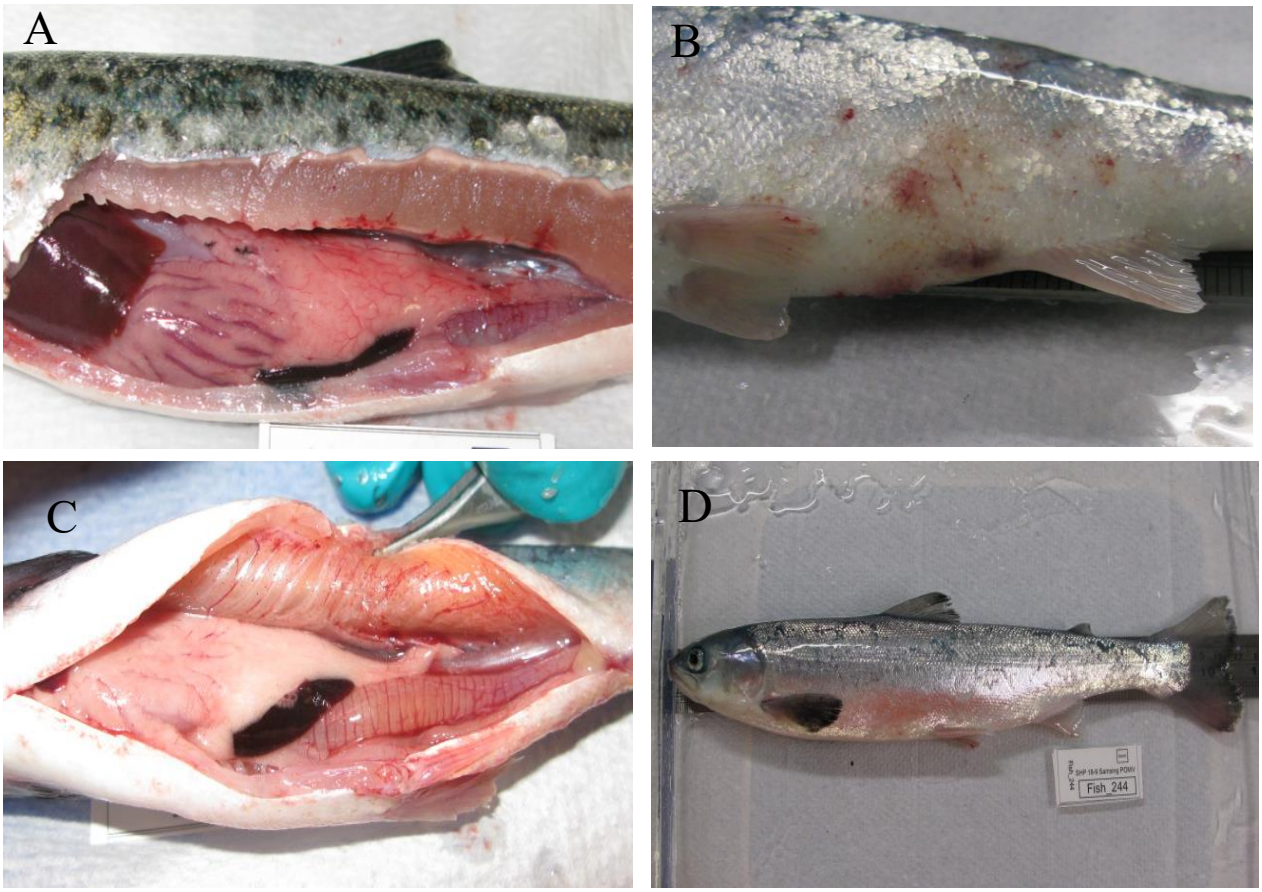


Figure 8. Moribund fish in laboratory-based Atlantic salmon infected with pilchard orthomyxovirus (POMV). Congestion and haemorrhages of pyloric caecae in cohab collected 12 dpi (A), haemorrhages around the vent in indirect collected at 12 dpi (B), splenogamy and mucus filled distal intestines in cohab collected at 15 dpi (C) and haemorrhages on ventral areas of the body in indirect collected at 11 dpi (Samsing, 2018).

The fish analysed in this study included sub-clinical fish defined as fish without any clinical signs of SON. We did not differentiate between cohabs and indirects but treated all of the samples "equal" by simply grouping them as sub-clinical fish. Moribund fish were only included in the study as positive controls for RT-qPCR and virus titration, and a few samples, together with naïve fish, were added for testing of the new RT-qPCR assays.

3.2 Viral tropism

The viral tropism was examined by detecting the viral load of POMV among different tissues and time points assessed using RT-qPCR (segment 5 assay) as relative quantification against ELF1α (log $2^{-\Delta\Delta Ct}$), virus titration ($TCID_{50}$) and immunohistochemistry on samples from

subclinical Atlantic salmon from the cohabitation challenge. Positive samples of POMV by virus titration showed characteristic POMV-like CPE with rounding and detachment of CHSE-cells (Mohr *et al*., 2020) (Fig. 9). Positive IHC samples, showed antigen staining in the tissue analyzed, ranging from one to several positive foci per field of view (Fig. 10).

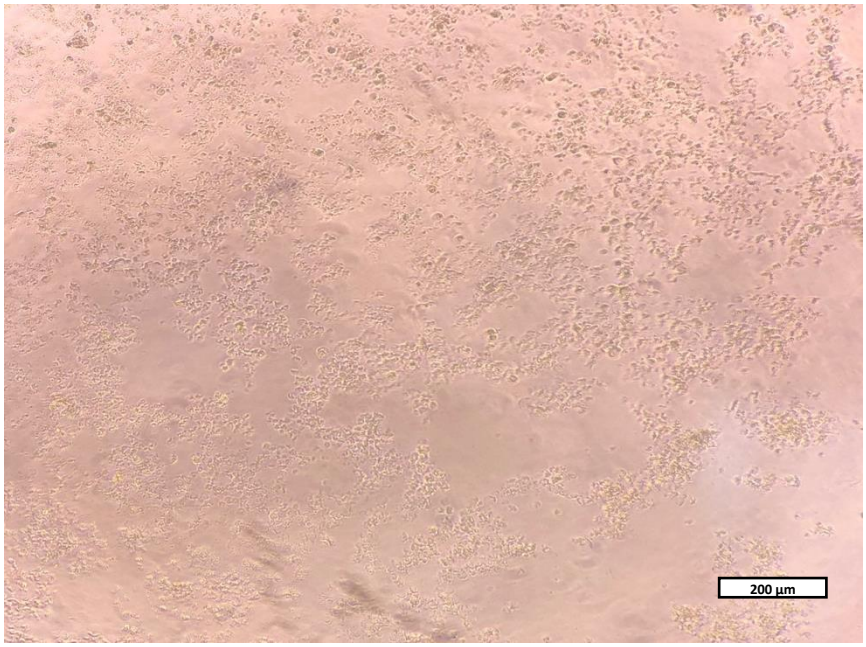


Figure 9. Clear sign of cytopathic effect due to POMV in CHSE-cells from moribund indirect fish (Atlantic salmon) sampled 15 days post injection.

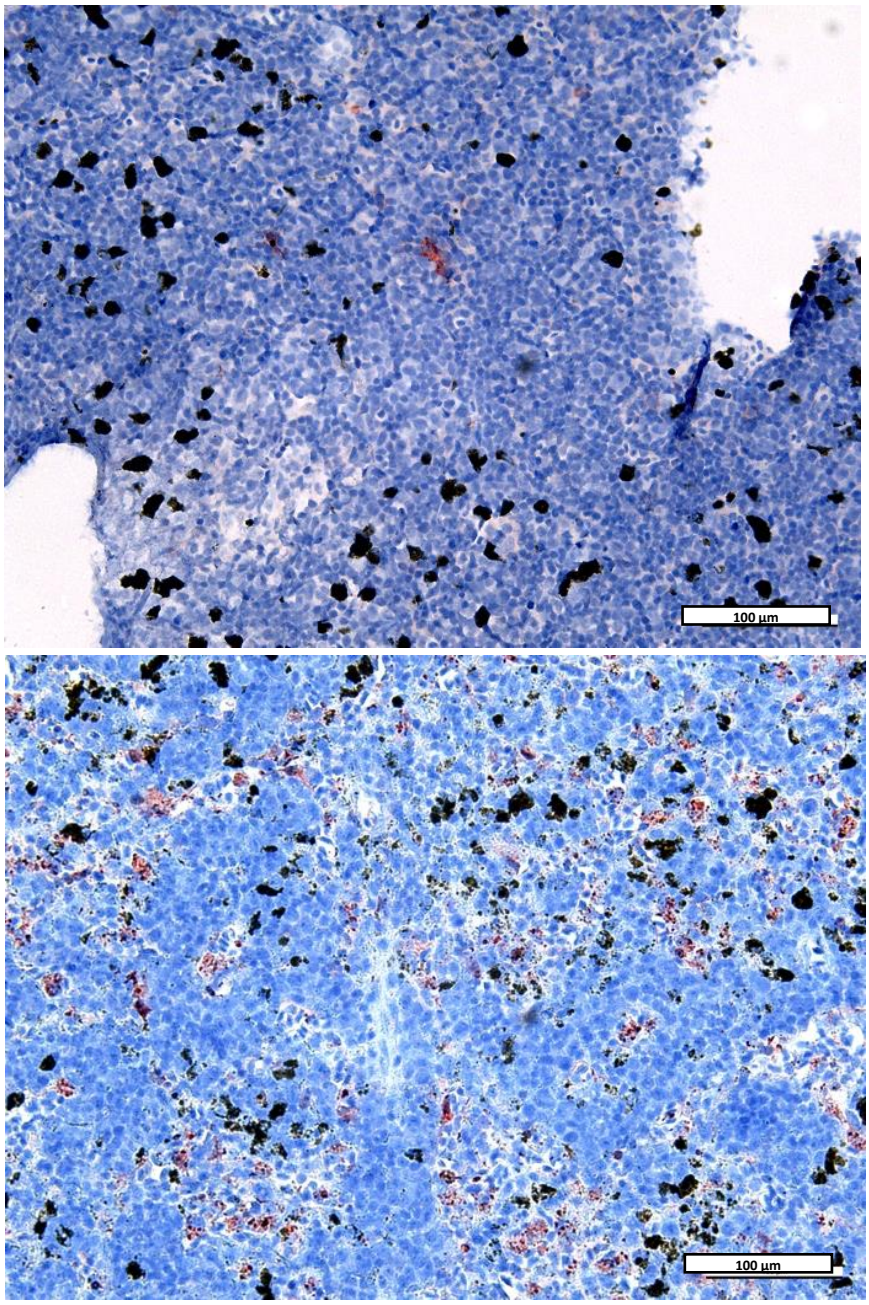


Figure 10. Immunohistochemistry (IHC) of POMV infected Atlantic salmon (*Salmo salar*) with anti-POMV antiserum. The upper figure show head kidney of a sub-clinical indirect fish sampled 2 post injection (dpi) with weak virus staining (score +). The lower figure show head kidney of moribund indirect fish sampled 15 dpi with a strong positive staining (score +++).

Analysis of RT-qPCR data revealed a strong interaction between tissue and time-point ($F_{25,128} = 5.8$, $p < 0.001$) on POMV loads (Fig. 11 A). Viral loads detected from virus titration increased during the experiment, with a significant effect of time ($F_{5,6}=5.1$, $p=0.04$), yet, not between tissues ($F_{2,34} = 1.0$, $p = 0.37$). For virus titration only head kidney, liver and spleen were tested (Fig. 11 B). However, similarly, analysis of viral loads by antigen staining from

IHC revealed a significant interaction between time-point (dpi) ($F_{5,20}$ = 4.0, p = 0.01) but not between tissues ($F_{1,4}$=1.4, P=0.31) (Fig. 11C). The interaction effect for fish type was also included in the Linear-mixed effects model, to summarize there was a significant difference between cohabs and indirects by virus titration, and evidence suggesting there was a similar, but, based on RT-qPCR and IHC, delayed infection in indirectly exposed fish. These results are further described in Samsing (*in press*).

Earliest detection of POMV was at 2 dpi with the highest viral levels in head kidney (IHC and RT-qPCR) followed by heart (RT-qPCR) (Fig. 11 A, B, C). By virus titration, the earliest detection was at 4 dpi in head kidney. The peak of infection, detected with all diagnostic methods, was between 8 and 12-dpi. At these points there was little difference in viral load between the different tissues (Fig. 11 A, B, C), supported by no significant difference between tissues for analysis of RT-qPCR data at 12 dpi ($F_{5,26}$= 0.2, p = 0.9). During late infection (19 dpi), highest viral load was found in the gill (IHC and RT-qPCR), head kidney (IHC), spleen (virus titration) and to a lesser extent in the heart (RT-qPCR) (post-hoc comparisons using Turkey's test $P < 0.05$).

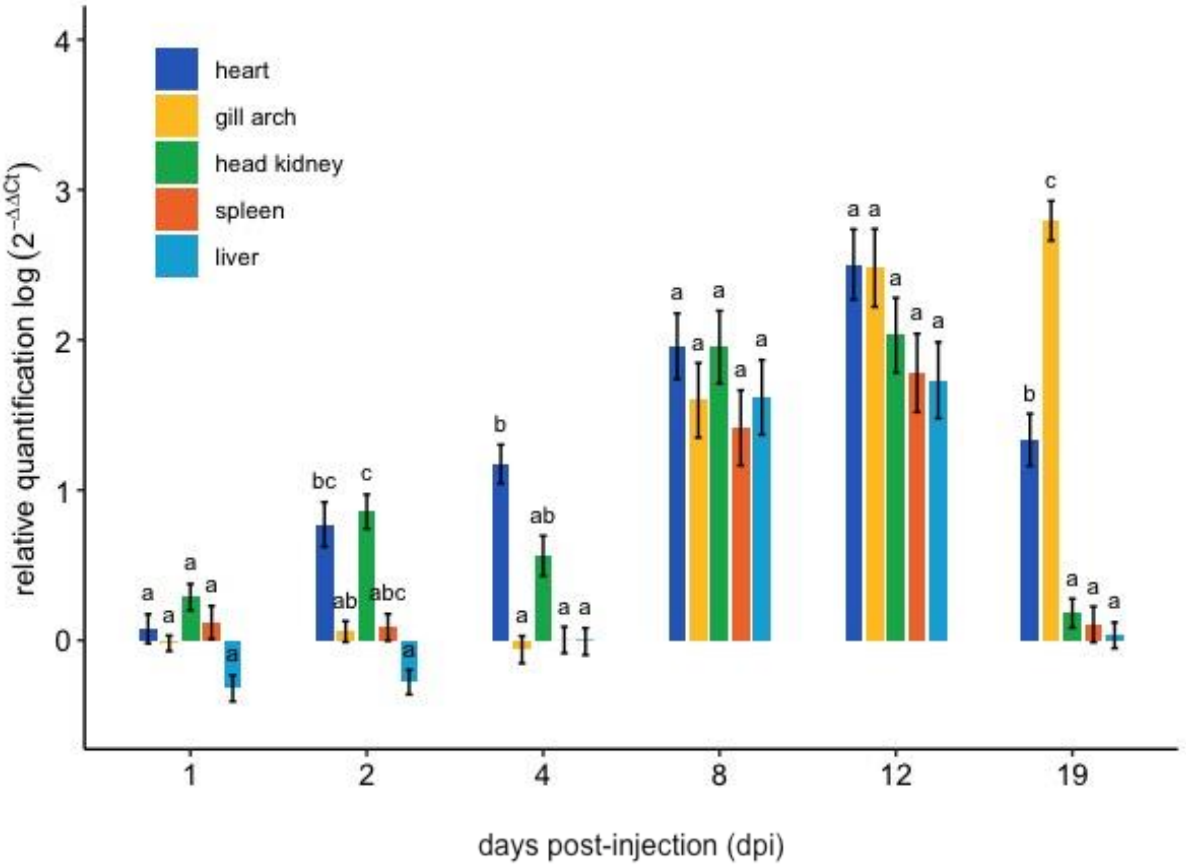


Figure 11 A. Pilchard orthomyxovirus (POMV) loads in six tissues at six time points from subclinical Atlantic salmon**.** Mean POMV load (±SE) per time point measured using the segment 5 POMV assay by RT-qPCR normalized against ELF1α (log $2^{-\Delta\Delta Ct}$). Within each time point (dpi), pairwise differences between means were assessed using Tukey's post-hoc comparisons. Different letters indicate significant differences ($p < 0.05$).

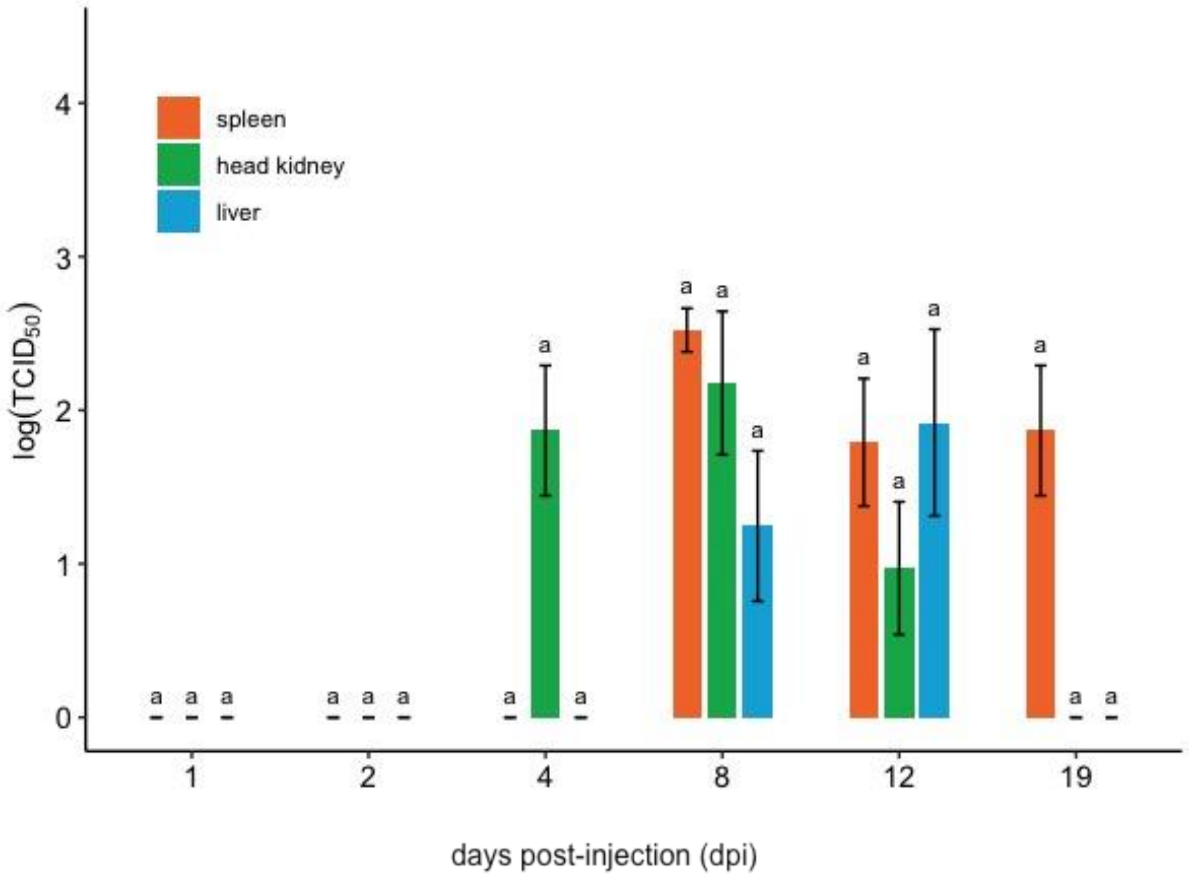


Figure 11 B. Pilchard orthomyxovirus (POMV) loads in three tissues at six time points from subclinical Atlantic salmon**.** Mean POMV load (±SE) per time point measured using virus titration. Within each time point (dpi), pairwise differences between means were assessed using Tukey's post-hoc comparisons. Different letters indicate significant differences ($p < 0.05$).

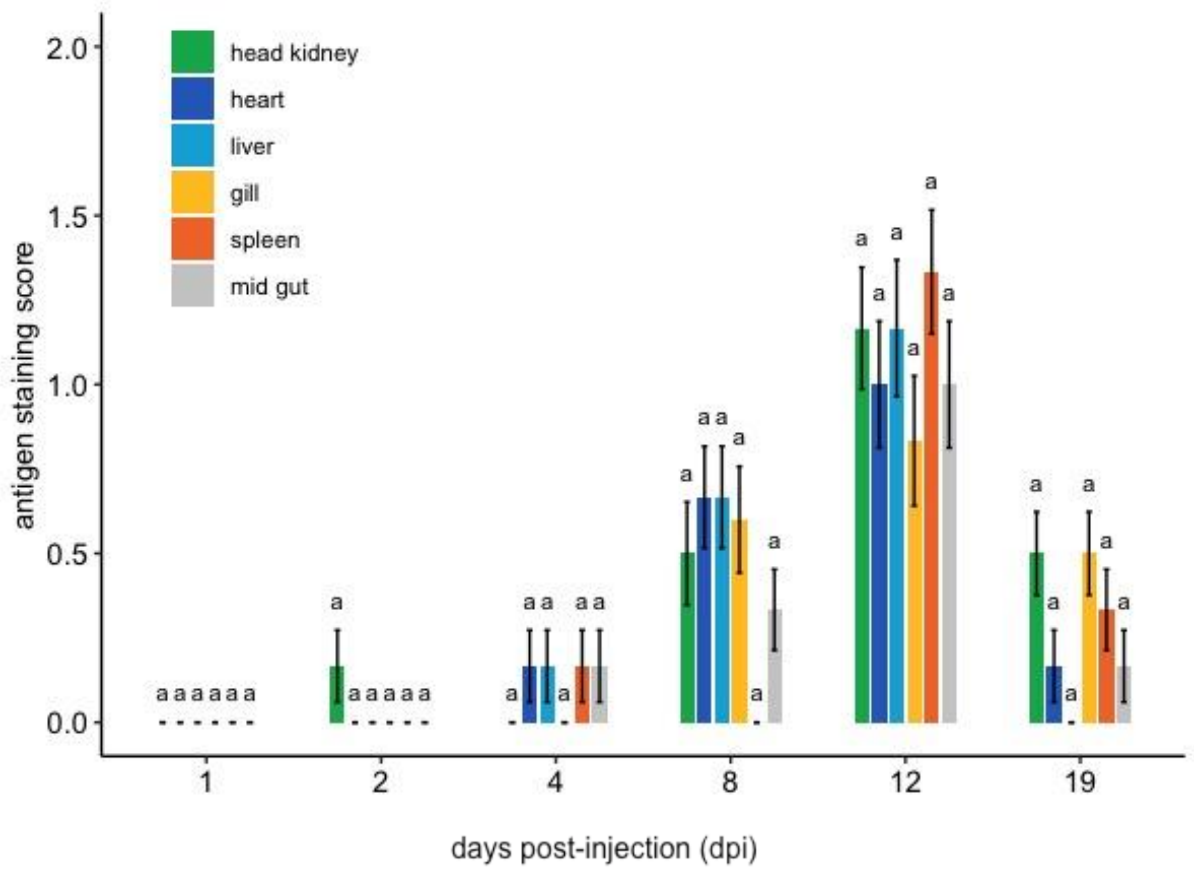


Figure 11 C. Pilchard orthomyxovirus (POMV) loads in six tissues at six time points from subclinical Atlantic salmon**.** Mean POMV load (±SE) per time point measured using antigen scores from immunohistochemistry. Within each time point (dpi), pairwise differences between means were assessed using Tukey's post-hoc comparisons. Different letters indicate significant differences ($p < 0.05$).

3.3 Reaction efficiency and Analytical sensitivity

Analytical sensitivity was assessed by determining the limit of detection (LOD) for the newly developed RT-qPCR assays. This was conducted using plasmid DNA with the respective amplicons for segment 7 and 8 inserted into a One Shot® TOP10 chemically competent *E. coli*. The results of these experiments are summarized in Table 7, and raw data is attached in Appendix 5. Both assays generated a linear standard curve, indicated by the correlation coefficient (R^2) value of 0.99 for both segment 7 and 8 assays. In addition, the standard curves generated an amplification efficiency of 100.62% for the segment 7 assay and 99.42% for the segment 8 assay. The highest dilution which provided Ct values for all four technical replicates determined the limit of detection (LOD) or the analytical sensitivity of the assay. The LOD was

deemed the Ct mean of 36.01± 0.88 for the segment 7 assay and 35.41± 1.09 for the segment 8 assay, equivalent to 6.80 and 5.48 plasmid copies/µl, respectively.

Table 7. Summary results from validation metrics for the segment 7 and 8 of POMV Taqman RT-qPCR assays.

	Segment 7	**Segment 8**
Standard curve R^2	0.99	0.99
Amplification efficiency (%)	100.62	99.42
Limit of detection (Ct mean ± SD)	35.41 ± 0.88	36.01 ± 1.09
Limit of detection (plasmid copies/µL)	6.80	5.48

3.4 Diagnostic sensitivity of the three different POMV assays

Diagnostic sensitivity (DSe) of the newly developed POMV assays were conducted using samples from the POMV challenge experiment. These included head-kidney samples from subclinical (n=26) and clinical/moribund fish (n=7). All three tests produced a positive result in 6 of 7 moribund (85.7%) fish, but this went down to only 7 of 26 (26.9%) in subclinical individuals. Meanwhile, 7 of 26 subclinical (26.9%) fish were positive to segment 8 only, and 4 of 26 (15.4%) to both segments 7 and 8 (Fig. 12).

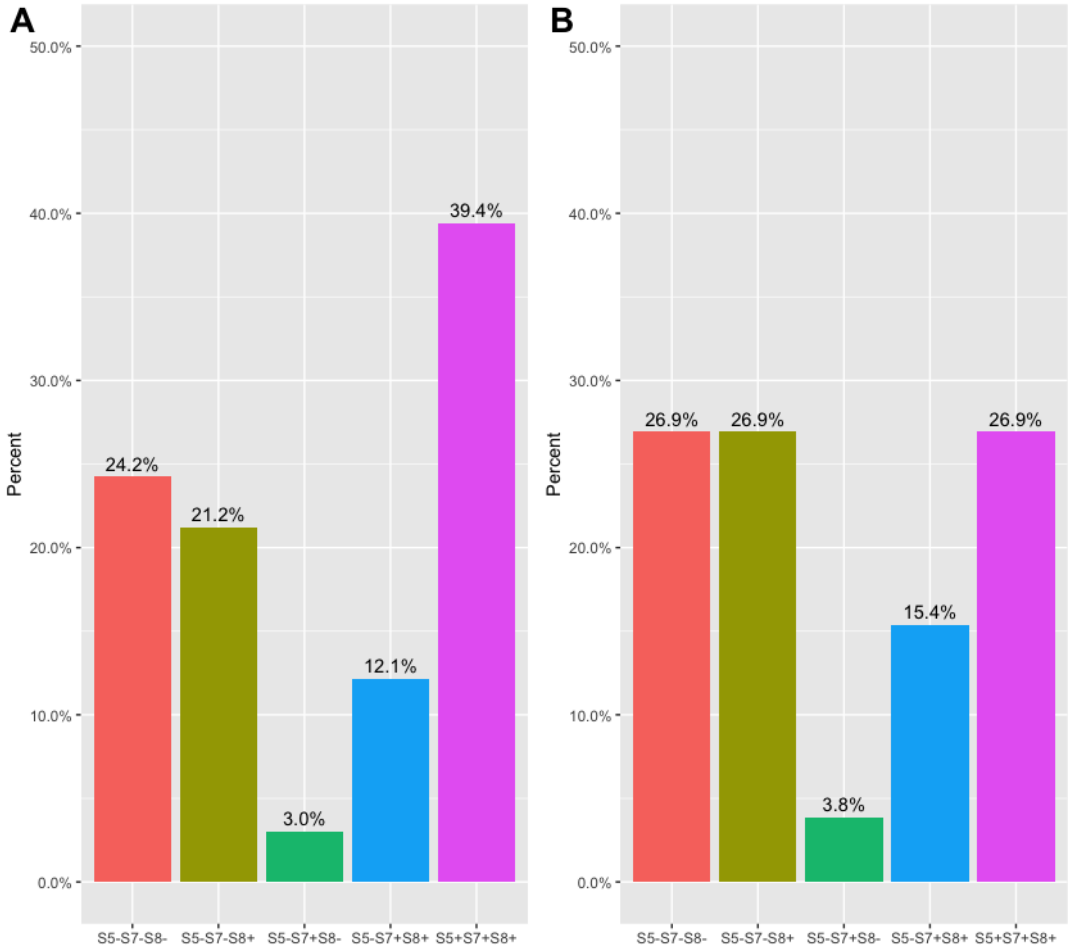


Figure 12. Percent of the samples that tested negative for all assays tested (S5-S7-S8-), positive for only the segment 8 assay (S5-S7-S8+), positive for only the segment 7 assay (S5-S7+S8-), positive for only the segment 7 and segment 8 assay (S5-S7+S8+), and positive for all assays tested (S5+S7+S8+). The samples tested were head kidney of moribund (n=7) and sub-clinical (n=26) fish from a cohabitation challenge. Figure A) include all samples (n=32) and B) sub-clinical samples only (n=26).

None of the existing reference methods for detection of POMV is considered diagnostically perfect (i.e. gold standard). Therefore, diagnostic sensitivity for each assay was estimated against a 'diseased' class, which included animals that were positive to either of the existing reference methods, namely RT-qPCR segment 5, virus titration, or IHC. If negative to all of these methods the individual was considered non-diseased. Two-by-two contingency tables (Table 8) with the counts of animals classified as diseased/non-diseased and positive/negative for segments 5, 7 and 8 were used to estimate diagnostic sensitivity for each assay. This estimation gave a DSe of 60% for the segment 5 assay 73 % for the segment 7 assay and 86.0 % for the segment 8 assay when both subclinical and clinical fish, and 100 % for all tests for clinically affected individuals. Pairwise comparisons showed a significant difference between

the sensitivity of the RT-qPCR against segment 5 and 8 (McNemar's test, $p = 0.01$), but no differences between segments 5 and 7 ($p = 0.08$) and between segments 7 and 8 ($p = 0.08$)

Tables 8 Two-by-two contingency tables with the counts of animals classified as diseased/non-diseased and positive/negative for segment 5, 7 and 8 assays for detection of POMV. The diseased class included animals that were positive to either RT-qPCR segment 5, virus titration or IHC (existing reference methods).

	Segment 5		
	Diseased	Non-diseased	Total
Test pos.	13	0	13
Test neg.	9	11	20
Total	22	11	33
	Segment 7		
	Diseased	Non-diseased	Total
Test pos.	16	2	18
Test neg.	6	9	15
Total	22	11	33
	Segment 8		
	Diseased	Non-diseased	Total
Test pos.	19	5	24
Test neg.	3	6	9
Total	22	11	33

Comparing Ct-values as relative quantification against ELF1α ($\log 2^{-\Delta\Delta Ct}$) using post-hoc Tukey's test showed a significant difference between segments 5 and 8 ($p < 0.001$), and segments 7 and 8 ($p < 0.001$), but no differences between segments 5 and 7 ($p = 0.18$). By looking at the samples in the light of the time they were collected there is larger differences between the assays at early (4 dpi) and late (19 dpi) infection stage (Fig 13). The linear mixed effect model also showed a significant difference between the state of the animal (sub-clinical/clinical fish, p=0.02), where a larger variation between the assays was observed for subclinical fish compared to clinical fish (Fig. 14).

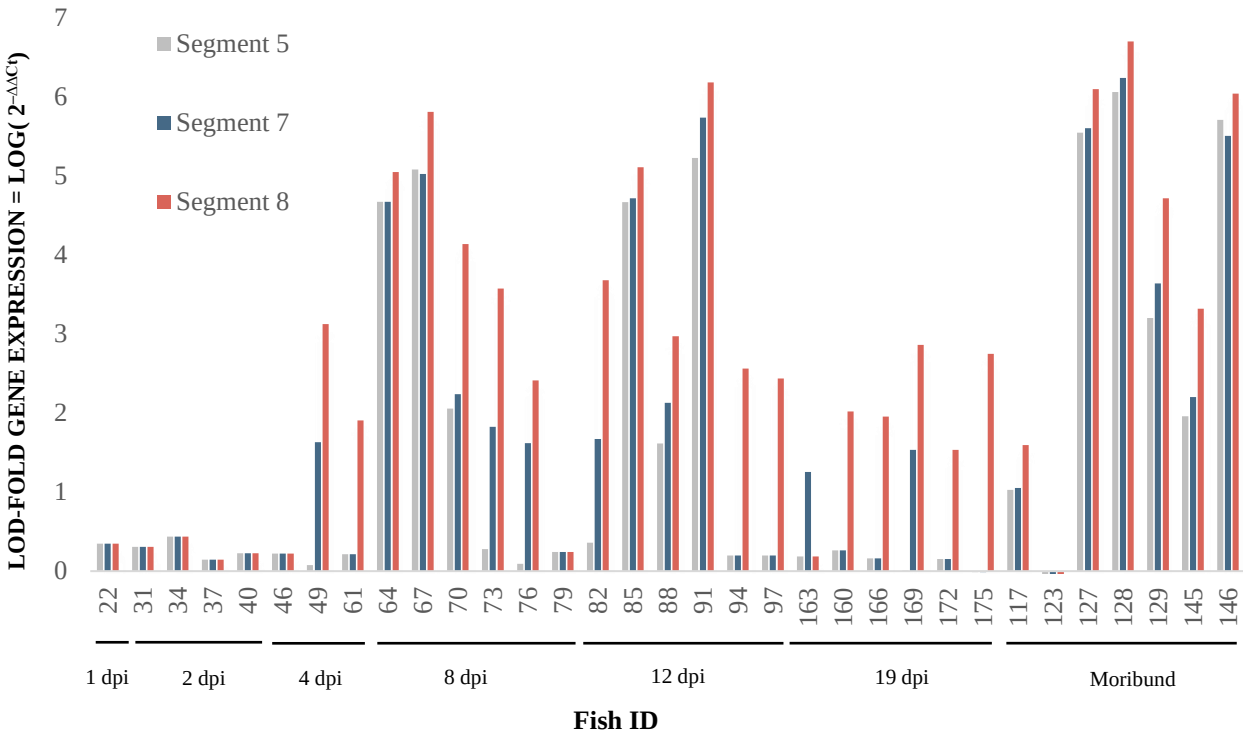


Figure 13. Head-kidneys collected from potential sub-clinical (n=26) and clinical/moribund (n=7) fish were tested to examine the sensitivity of the POMV assays, targeting segment 5, 7 and 8. The transcript levels are normalized against Elf1α mRNA (log $2^{-\Delta\Delta Ct}$).

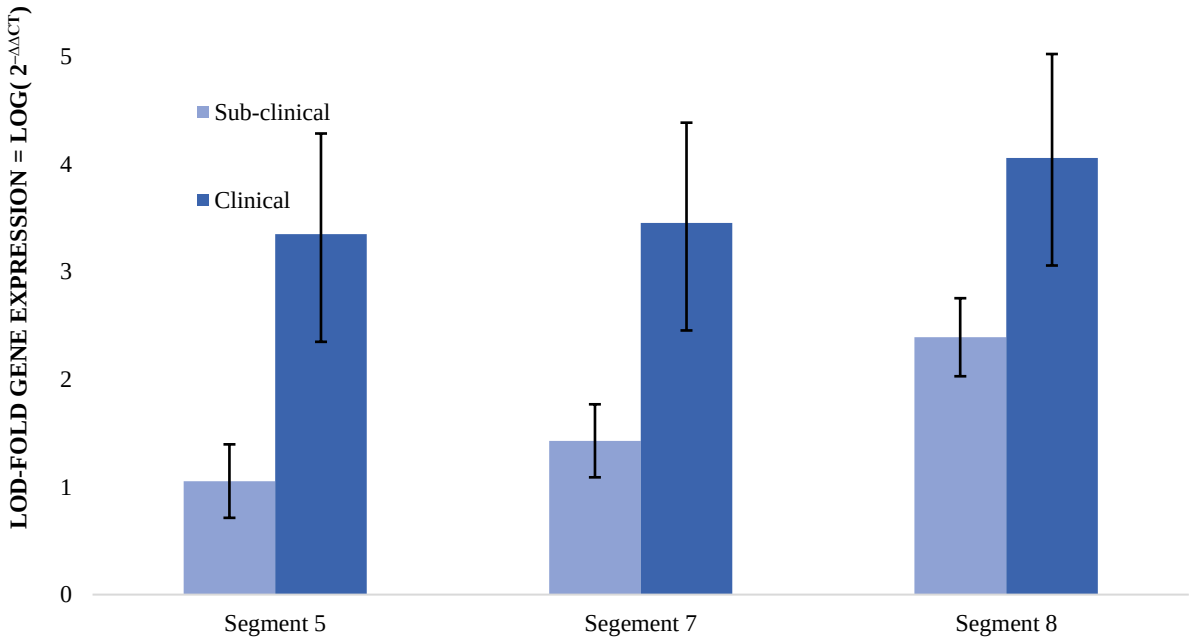


Figure 14. Head-kidneys collected from potential sub-clinical (n=26) and clinical/moribund (n=7) presented as an average of the individual transcript levels for each fish with mean + SEM. The transcript levels are normalized against Elf1α mRNA.

3.5 Diagnostic specificity of different POMV assays

Samples of naïve fish (pre-challenge negative controls, n=5) were used to investigate diagnostic specificity (DSp) of the three assays. All samples tested negative for POMV, and therefore the estimate of diagnostic specificity was 100% for all assays.

4. Discussion

The present study describes the tissue tropism of a novel orthomyxovirus, Pilchard orthomyxovirus (POMV), and the development of a new diagnostic assay for its early detection. POMV was detected in all tissues in various quantities at different time points, improving the understanding of the tissue distribution of the virus. This knowledge, together with the newly developed sensitive and specific Taqman RT-qPCR assays targeting segment 7 and 8 of the viral RNA can contribute to earlier detection of infection due to POMV. The currently available RT-qPCR assay targeting segment 5 of the genome has previously shown to successfully diagnose Atlantic salmon showing clinical signs of disease (Mohr et al., 2020). However, the assays developed in this study show an improved sensitivity for POMV when tested on sub-clinical fish, and can possibly improve management and reduce losses due to SON in Tasmania.

4.1 POMV presence in different tissues

POMV was consistently detected at early time-points (2-4 dpi) in head-kidney and heart by all the methods conducted (Fig. 11, A, B, C). This finding suggest that these tissues may be initial site of infection and therefore effective samples for early detection of POMV. These were also among the tissues with highest viral loads throughout the time course (Fig. 11 A, B, C), supporting that these are important sites for POMV infection. Similarly, ISAV is detected in these tissues at early time points and presents high viral loads during infection, however, gills are the first tissue ISAV is detected (Dannevig et al., 1994; Totland et al., 1996; Rimstad *et al.*, 1999; Mikalsen *et al.*, 2001; Austbø *et al.*, 2014). This is not surprising, as the gills are in constant contact with the water and the detection could be due to presence of ISAV in the water binding to the gills. However, replication has been detected by the use of primers and probe targeting the transcript junction of segment 7 of the viral genome which is only present

when the virus is replicating (Austbø et al., 2014). In addition, several studies have shown early replication in endothelial cells in the gills, and it appears to be the main port of entry for ISAV (Aamelfot et al., 2012; Austbø et al., 2014; Mikalsen et al., 2001; Totland et al., 1996; Weli et al., 2013). This does not exclude other routs of infection, as ISAV is detected and shown to replicate in other mucosal surfaces such as pectoral fins, skin and gastrointestinal track (Aamelfot et al., 2015) which could be potential portals for POMV infection as well. Our results indicate that gill tissue may not be the main route of infection for POMV, as low levels of virus is detected during early infection. An alternatively explanation is that there is a transient epithelial infection in the gills, not detectable with the current RT-qPCR assay or IHC which is followed by viremia and higher replication in other tissues.

Our results imply that the target cells for POMV are present in all or most tissues (Fig. 11 A, B, C) which match with the multisystemic pathology associated with SON (Samsing et al., *in press*; Godwin et al., 2020; Morrison et al., 2013; Godwin *et al.*, 2016). This finding also correlates with the suggested target cell of POMV (Samsing et al., *in press*; Godwin et al., 2020; Morrison et al. 2013) and also commonly regarded target cell for other orthomyxoviruses (Hovland et al 1994; Nylund et al 1995b; Koren & Nylund 1997; Nylund et al 1996, 1997; Aamelfot et al. 2012; Subbarao et al. 1998), endothelial cells, as they line blood vessels in all organs. During the time course, a general trend was observed where viral loads increased over time with a peak between 8 and 12 dpi followed by a reduction in viral loads (Fig. 11 A, B, C). For head kidney, viral RNA detected by RT-qPCR reached a peak at 12 dpi and followed by a decrease at 19 dpi (Fig. 11 A). A similar pattern was observed for virus titration, however there was more infectious viruses at 8 than 12 dpi, suggesting early replication in this tissue.

Viral RNA in the gills did not follow a similar trend as seen for the other tissues, in contrast viral loads continued to increase towards the end of the trial. This detection could be a result of accumulation of viruses in the water and virus binding to the gills. It could also suggest that POMV persist in the gill for a longer time than in other organs and could be shedding from this tissue, as proposed for ISA (Aamelfot et al., 2015). Viral shedding could also be occurring from other tissues as shown for ISA, including skin, mucus, faeces and urine (Nylund et al., 1994; Totland et al., 1996). The gastrointestinal tract has been suggested to be an important tissue for viral shedding of POMV (Godwin et al, 2020) as accumulation of mucus in this tissue is a common pathology trait for SON (Godwin et al., 2020, Samsing et al,

in press) and was also a prevalent finding in this experiment. In support of this POMV has also been isolated form the mucus from the gastrointestinal tract (Godwin et al, 2020).

POMV follow similar patterns as seen for challenges with ISAV (Godwin et al., 2020), where fish that are directly exposed to the virus (by immersion or IP injection) develop disease sooner than fish exposed by cohabitation, e.g (Wolf et al., 2013). In support of this, mortalities from our cohabitation challenge were detected at 4 dpi for trojans and at 6 and 7 dpi for cohabs and indirects, respectively (Fig. 7). Samsing (*in press*) show evidence suggesting a similar, but delayed infection in indirectly exposed fish compared to cohab fish by IHC and RT-qPCR. POMV develop disease more rapidly than ISAV (Godwin et al.,2020) which is also supported by the results of our experiment (Fig. 7). In IP-experiments mortalities for ISA is detected 10-20 days post-exposure by IP-injection with cohabs mortalities 10 days later (Caruffo et al., 2016; Jones & Groman, 2001; Raynard et al., 2001; Wolf et al., 2013). In contrast, POMV mortalities was first detected in cohabs 7 days post-exposure by IP-injection of trojans (Godwin et al., 2020).

Our results suggest that POMV can be detected at earlier time points than ISAV. By RT-qPCR and IHC several samples tested positive at 2 dpi, however POMV was not detected replicating until 4 dpi (Fig. 11 A, B, C). In immersion experiments, replicating ISAV has been detected as early as day 2 after addition of virus in the water (Austbø et al., 2014). However, in cohabitation experiments ISAV has been detected at 5-13 dpi (Mikalsen et al., 2001; E Rimstad et al., 1999). By cohabitation, it has also been shown that ISAV have a delayed peak in viral load compared to POMV, as ISAV has been detected with highest viral loads at approximately 15 dpi (Mikalsen et al., 2001; Rimstad et al., 1999). Described by Mikalsen (2001), this peak was followed by a decrease in viral load, however, after 25 dpi a second rise was detected which continued to the terminal stage of the experiment (70 dpi). A similar delayed peak was not shown for POMV, and our results might indicate that ISAV has a slower and more long-lasting infection compared to POMV. However, the cohabitation challenge was terminated at 19 dpi so further development of infection at later time points cannot be excluded.

4. 2 Assay development and test accuracy

Recent gene expression data indicate that other segments of the viral genome of POMV could be better candidates for early detection of POMV, than the currently used assay targeting segment 5 (Samsing et al, *in press*). To improve sub-clinical detection of POMV other potential targets of its genome were explored. The segment 7 and segment 8 were chosen based on evidence of high expression of the two segments early in the infection (6- and 24-hours post infection) by next generation sequencing data in ASK-cells, kidney and liver (Samsing et al, *in press*). In addition, these segments are targeted by the recognised method for detecting ISAV by RT-PCR (Snow et al., 2006, OIE 2019) commonly used during disease outbreak and in apparently healthy fish (OIE, 2019).

The segment 7 and segment 8 assays were shown to be robust as concentration changes of primers and probes gave minimal variation in Ct-values and low SDs were generated (Appendix 4). The assays tested positive for a range of different concentrations from 10^7 to 10 plasmid copies containing the target sequence with amplification efficiencies close to 100%. Similar analytic sensitivities were shown for segment 5, 7 and 8 assays with LODs <10 plasmid copies/ul (Mohr et al, 2020). This indicates that the PCR amplification in the assays are near optimal and good candidates for quantification of RNA in samples. However, no RT-PCR assays can be used to quantify actual viral particles in the tissue as the RNA present includes mRNA, genomic RNA and antigenomic RNA (complementary RNA) (Fig. 4).

The three assays tested accurately for all samples with known animal state, negative to all negative controls, thereby shown to have 100% diagnostic specificity, and positive for all clinical fish. The assay targeting segment 8 had the highest diagnostic sensitivity of all three assays, followed by segment 7 and 5, respectively. This finding was supported by the consistent detection of the higher Ct-levels by the segment 8 assay followed by the segment 7 assay and then segment 5 assay in experimental infected fish, with only a few exceptions. Since the segment 7 and 8 assays display similar theoretical efficiencies and also similar LODs to segment 5, such finding is most likely related to the differential expression of the segment throughout the infection cycle. An increased abundance of segment 7 and 8 mRNA generated during the viral cycle, especially during early and late infection (Fig. 13), might

explain the apparent improved sensitivity. This is supported by larger variation between the assays for the sub-clinical fish compared to clinical fish (Fig. 14).

POMV segment 5 encodes for the acidic polymerase protein (PA) (Mohr et al., 2020), an important component of the RNA polymerase complex and therefore essential for further transcription of viral proteins (Aspehaug et al., 2004; Falk et al., 2004; Goić et al., 2008; Ritchie et al., 2001b). The proteins encoded by segment 7 and 8 are not known, as they do not align with proteins of ISAV or other orthomyxoviruses (Mohr et al., 2020). POMV has two possible ORFs for segment 7 and one for segment 8 (Mohr et al., 2020), in contrast to ISAV which has two ORFs for each of the segments which encode; IFN antagonist (NS1), nuclear export protein, matrix protein 1 and another IFN antagonist (NS2), respectively (Biering et al., 2002; Falk et al., 2004; García-Rosado et al., 2008; Kibenge et al., 2007; Li et al., 2016; Mcbeath et al., 2006). Both POMV and ISAV are shown to induce significant innate antiviral responses such as early up-regulation of host pathogen recognition receptors, RIG-I and TLR3 producing type I IFNs which play a crucial role in the first line of defence against viruses in vertebrates (Samsing et al., 2019; Svingerud et al., 2013). It is therefore plausible that, similar to ISAV, POMV segment 7 and/or 8 may encode for interferon antagonists as also seen for other orthomyxoviruses, and this may explain the early detection of these segments (Biering et al., 2002; García-Rosado et al., 2008; Li et al., 2016; Mcbeath et al., 2006).

The POMV segment 7 and 8 assays also showed a positive result for samples that were negative for all available reference tests (RT-qPCR for POMV segment 5, virus titration and IHC) (Table 8). The samples that *only* tested positive for either the segment 7 and/or 8 assays presented higher Ct-values, suggesting lower viral loads in these tissues. This could explain why this samples were negative to viral titration and IHC. These samples were also from early and late infection stages were the segment 7 and 8 assays were shown to generate the largest differences in Ct compared to segment 5. This indicates that these assays might be more useful for testing sub-clinical infection and that the samples may reflect either a carrier status of SON, pre-viraemia, or post-viraemia where the host is in the process of clearing the virus (Hodneland et al., 2006).

4.3 Application of a more sensitive diagnostic test

A sensitive diagnostic tool will be able to detect presence of POMV infections at early time points which is important regarding control and eradication of the virus. SON is thought to have a more rapid disease development than ISA (Mohr et al, 2020), yet little is known about SON's disease characteristics. ISA is shown to be a slow developing disease, and can be present with few clinical signs and with low mortalities which can be easily overlooked (Rimstad et al., 2011). By identifying infected individuals at this phase, hosts can be eliminated from the population and further transmission can be prevented. This could be of great importance to control transmission of POMV as the virus seems to transmit and be highly infectious thought the water column (Samsing et al, *in press*). For almost all infectious diseases dose, virus strain, environmental factors and genetic make-up of the host will influence disease development (Rimstad et al., 2011). These variables also apply to ISA, and the disease can also occur in episodes of acute high mortalities with more severe pathology (Thorud & Djupvik, 1988), especially if no measures are taken (Rimstad et al., 2011). An increased mortality seems to be related to stress situations for both ISAV and POMV (Rimstad et al., 2011; Galea et al., 2018). Outbreaks of SON seems to be related to stress such as low oxygen, high water temperatures and possibly other factors which is not understood (Galea et al., 2018). Early detection of the virus can therefore be important for in-farm management as such stress factors may be avoided and thereby possibly decrease losses and improve the animal welfare.

4.4 Further steps

As described in this thesis, the segment 7 and 8 assay have been designed, optimized and validated in several steps. OIE describes these steps and a set of other principles and methods to be fulfilled in order to achieve a validated assay (Fig. 15). Our experiment has successfully completed the assay development pathway in addition to the analytical and diagnostic characteristics (step 1 and 2) of the assay validation pathway (Fig. 15). However, analytic specificity was only tested *in silico*. Currently, CSIRO are working on testing actual analytical specificity (inclusivity and exclusivity) of the new POMV assays. For testing exclusivity, which is the capacity of an assay to detect an analyte or genomic sequence that is unique to a target organism, the assay will be tested against exotic (ISAV, RbtOV) and

endemic (TRSV 1 and 2, TAB) viral pathogens of Atlantic salmon. It is of importance that the two Tasmanian viruses, Tasmanian Aquabirnavirus (TABV) and Tasmanian salmon Aquareovirus (TSRV), are tested as they represent the relevant viruses affecting salmonids in Tasmania (Huon Aquaculture, 2017). Meanwhile, ISAV and Rbtov are exotic to Australia (Department of Agriculture, 2019), but they are the only viruses affecting salmonids that belong to the same family as POMV, *orthomyxoviridae*, (International Committee on the Taxonomy of Viruses, 2020) and could therefore contain similar nucleotide sequences. If these viruses were spread to Australia, it would be of importance that they were not detected by the assay as POMV. For inclusivity testing, which is the capacity of an assay to detect different strains or isolates, the newly developed POMV assays will be tested against all 11 isolates of POMV present in Australia (Mohr et al. 2020).

According to OIE (2010), the next step in the assay validation pathway is reproducibility. Interlaboratory reproducibility will be conducted in parallel at the CSIRO, Department of Primary Industries in Tasmania (DPIPWE) and the Australian Animal Health Laboratory (AAHL) in Victoria, Australia. This step is important to ensure consistent results among different laboratories and will be performed on 30 field samples, using approximately 20 positive samples (with a wide range of Ct values) and 10 known negative samples.

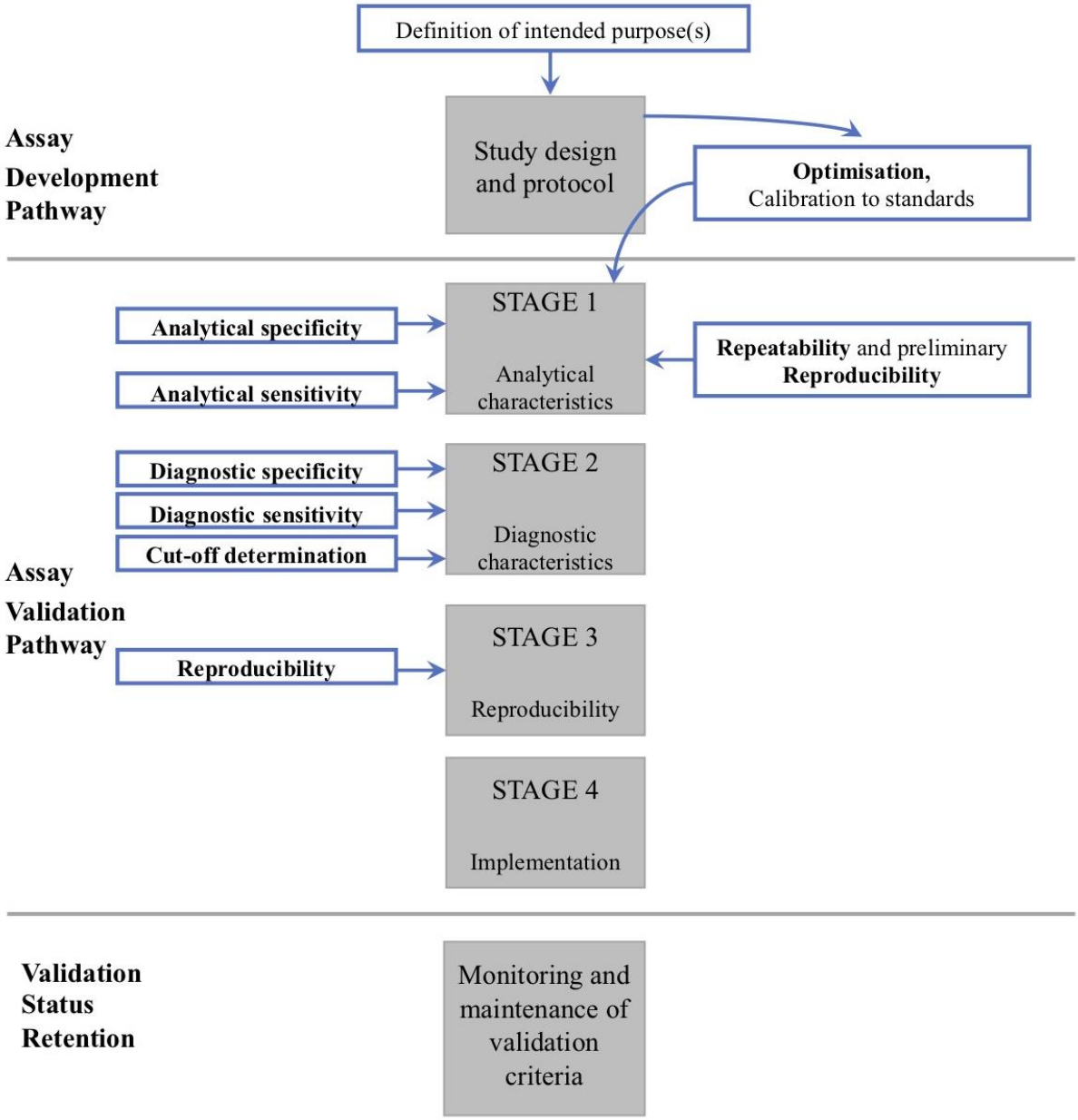


Figure 15. The assay development and validation pathways with assay validation criteria highlighted in bold typescript within the boxes, adapted from OIE (2010).

In a field perspective, it will be of importance to differentiate between vaccinated and diseased fish as Tasmanian Atlantic salmon are currently being vaccinated against SON (Norwood, n.d.). There are two standard vaccine technologies used in aquaculture, inactivated and recombinant vaccines. These contain either inactivated microorganisms, or subunits on an organisms, and can induce an immune response in the host without the ability to replicate in the host's cells (Berg et al., 2006). At present, neither the newly designed assays nor the POMV segment 5 assay have the ability to differentiate vaccinated and diseased fish as they detect presence of viral RNA. By targeting a spliced variant of the genome only infectious

replicating viruses could be detected, as the assay will be limited to target mRNA only. Such a diagnostic test would therefore be desirable to accurately diagnose a vaccinated population. Such a tool could also provide further information about tissue tropism such as initial sites of replication, ports of entry, and shedding routes of the virus.

4.5 Conclusion

Tissue tropism of POMV was examined by RT-qPCR segment 5 assay, virus titration and IHC through a time course cohabitation challenge with IP-injected Atlantic salmon. POMV was detected in all six tissues examined in sub-clinical fish, head kidney and heart being the first sites to show presence of POMV two days after infection. A peak of infection was detected between 8- and 12-days post injection in all organs, with the exception of the gills which presented high viral loads past this point. The designed qRT-PCR assays targeting segment 7 and 8 of POMV were validated and shown specific and sensitive through several steps. The improved diagnostic sensitivity of the assays, especially for the segment 8 assay, will be particularly important as it can contribute to earlier detection of the virus and improve surveillance testing of POMV in the field and implementations of preventive strategies to control the disease.

References

Aamelfot, M., Dale, O. B., & Falk, K. (2014). Infectious salmon anaemia - pathogenesis and tropism. *Journal of Fish Diseases*, *37*(4), 291–307. https://doi.org/10.1111/jfd.12225

Aamelfot, Maria. (2013). *Tropism of infectious salmon anaemia virus and distribution of the 4-O-acetylated sialic acid receptor*. Norwegian School of Veterinary Science Oslo.

Aamelfot, Maria, Dale, O. B., Weli, S. C., Koppang, E. O., & Falk, K. (2012). Expression of the Infectious Salmon Anemia Virus Receptor on Atlantic Salmon Endothelial Cells Correlates with the Cell Tropism of the Virus. *Journal of Virology*, *86*(19), 10571 LP – 10578. https://doi.org/10.1128/JVI.00047-12

Aamelfot, Maria, Mcbeath, A., Christiansen, D. H., Matejusova, I., & Falk, K. (2015). Infectious salmon anaemia virus (ISAV) mucosal infection in Atlantic salmon. *Veterinary Research*, *46*(1), 120. https://doi.org/10.1186/s13567-015-0265-1

Agilent technologies. (2012). *Introduction to Quantitative PCR - Methods and Applications Guide*. *1*, 104. https://doi.org/10.1111/j.1472-765X.2009.02749.x

AgPath-ID One-Step RT-PCR Reagents. (n.d.). *1005*.

Animal Health Committee (AHC). (2018). *Australia's National List of Reportable Diseases of Aquatic Animals*. https://www.agriculture.gov.au/animal/aquatic/reporting/reportable-diseases#finfish

Applied Biosystems. (2002). *Data Analysis on the ABI P RISM ® 7700 Sequence Detection System : Setting Baselines and Thresholds Overview*.

Aslam R W, Bates V, Dundar Y, Hounsome J, Richardson M, Krishan A, Dickson R, Boland A, Kotas E, Fisher J, S. S. & R. L. (2016). Automated tests for diagnosing and monitoring cognitive impairment: a diagnostic accuracy review. In *Health Technology Assessment* (Vol. 20, Issue 77). https://doi.org/https://doi.org/10.3310/hta20770

Aspehaug, V., Falk, K., Krossøy, B., Thevarajan, J., Sanders, L., Moore, L., Endresen, C., & Biering, E. (2004). Infectious salmon anemia virus (ISAV) genomic segment 3 encodes the viral nucleoprotein (NP), an RNA-binding protein with two monopartite nuclear localization signals (NLS). *Virus Research*, *106*(1), 51–60.

Aspehaug, V., Mikalsen, A. B., Snow, M., Biering, E., & Villoing, S. (2005).

Characterization of the infectious salmon anemia virus fusion protein. *Journal of Virology*, *79*(19), 12544–12553.

Assefa, A., & Abunna, F. (2018). Maintenance of Fish Health in Aquaculture: Review of Epidemiological Approaches for Prevention and Control of Infectious Disease of Fish. *Veterinary Medicine International*, *2018*, 5432497. https://doi.org/10.1155/2018/5432497

Austbø, L., Aas, I. B., König, M., Weli, S. C., Syed, M., Falk, K., & Koppang, E. O. (2014). Transcriptional response of immune genes in gills and the interbranchial lymphoid tissue of Atlantic salmon challenged with infectious salmon anaemia virus. *Developmental and Comparative Immunology*, *45*(1), 107–114. https://doi.org/10.1016/j.dci.2014.02.007

Batts, W. N., LaPatra, S. E., Katona, R., Leis, E., Ng, T. F. F., Brieuc, M. S. O., Breyta, R. B., Purcell, M. K., Conway, C. M., Waltzek, T. B., Delwart, E., & Winton, J. R. (2017). Molecular characterization of a novel orthomyxovirus from rainbow and steelhead trout (Oncorhynchus mykiss). *Virus Research*, *230*, 38–49. https://doi.org/10.1016/j.virusres.2017.01.005

Berg, A., Fjelldal, P. G., & Hansen, T. (2006). Fisken og havet: Dyrevelferdsmessige konsekvenser av vaksinasjon av fisk - effekter og bivirkninger. *Havforskningsinstituttet*, *9*.

Biering, E., Falk, K., Hoel, E., Thevarajan, J., Joerink, M., Nylund, A., Endresen, C., & Krossøy, B. (2002). Segment 8 encodes a structural protein of infectious salmon anaemia virus (ISAV). *Diseases of Aquatic Organisms*, *49*, 117–122. https://doi.org/10.3354/dao049117

Bouchard, D. A., Brockway, K., Giray, C., Keleher, W., & Merrill, P. L. (2001). First report of infectious salmon anemia (ISA) in the United States. *BULLETIN-EUROPEAN ASSOCIATION OF FISH PATHOLOGISTS*, *21*(2), 86–88.

Cadwallader, P. L. (1996). *Overview of the impacts of introduced salmonids on Australian native fauna*. Australian Nature Conservation Agency.

Caruffo, M., Maturana, C., Kambalapally, S., Larenas, J., & Tobar, J. A. (2016). Protective oral vaccination against infectious salmon anaemia virus in Salmo salar. *Fish & Shellfish Immunology*, *54*, 54–59. https://doi.org/10.1016/j.fsi.2016.03.009

Cler, J. P. M., Fuller, F., & Bishop, D. H. L. (1983). Tick-borne viruses structurally similar to

orthomyxoviruses. *Virology*, *127*(1), 205–219. https://doi.org/10.1016/0042-6822(83)90384-7

Cottet, L., Rivas-Aravena, A., Cortez-San Martin, M., Sandino, A. M., & Spencer, E. (2011). Infectious salmon anemia virus--genetics and pathogenesis. *Virus Research*, *155*(1), 10–19. https://doi.org/10.1016/j.virusres.2010.10.021

Crane, M., & Hyatt, A. (2011). *Viruses of fish: an overview of significant pathogens. Viruses 3: 2025–2046*.

CSIRO. (2016). Atlantic Salmon Breeding Case Study. In *STRATEGY, MARKET VISION AND INNOVATION*.

Dannevig, B H, Falk, K., & Skjerve, E. (1994). Infectivity of internal tissues of Atlantic salmon, Salmo salar L., experimentally infected with the aetiological agent of infectious salmon anaemia (ISA. *Journal of Fish Diseases*, *17*(6), 613–622. https://doi.org/10.1111/j.1365-2761.1994.tb00259.x

Dannevig, Birgit H, Falk, K., & Namork, E. (1995). Isolation of the causal virus of infectious salmon anaemia (ISA) in a long-term cell line from Atlantic salmon head kidney. *Journal of General Virology*, *76*(6), 1353–1359.

Department of Agriculture. (2019). *Australian aquatic veterinary emergency plan (AQUAVETPLAN) for infectious salmon anaemia*.

Department of Primary Industries Parks Water and Environment. (2016). *Sustainable industry growth plan for the salmon industry*.

Desjardins, P., & Conklin, D. (2010). NanoDrop Microvolume Quantitation of Nucleic Acids. *Journal of Visualized Experiments : JoVE*, *45*. https://doi.org/10.3791/2565

Devold, M., Karlsen, M., & Nylund, A. (2006). Sequence analysis of the fusion protein gene from infectious salmon anemia virus isolates: evidence of recombination and reassortment. *Journal of General Virology*, *87*(7), 2031–2040.

Dias, A., Bouvier, D., Crépin, T., McCarthy, A. A., Hart, D. J., Baudin, F., Cusack, S., & Ruigrok, R. W. H. (2009). The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. *Nature*, *458*(7240), 914–918.

Eliassen, T. M., Frøystad, M. K., Dannevig, B. H., Jankowska, M., Brech, A., Falk, K., Romøren, K., & Gjøen, T. (2000). Initial events in infectious salmon anemia virus

infection: evidence for the requirement of a low-pH step. *Journal of Virology*, *74*(1), 218–227.

Endmemo. (n.d.). *DNA/RNA Copy Number Calculator*. Retrieved November 23, 2019, from http://endmemo.com/bio/dnacopynum.php

English, C., Swords, F., Downes, J., Ruane, N., Botwright, N., Taylor, R., Barnes, A., Wynne, J. W., Lima, P., & Cook, M. T. (2019). Prevalence of six amoeba species colonising the gills of farmed Atlantic salmon with amoebic gill disease (AGD) using qPCR. *Aquaculture Environment Interactions*, *11*. https://doi.org/10.3354/aei00325

European Commission. (2006). Council Directive 2006/88/EC on animal health requirements for aquaculture animals products thereof, and on the prevention and control of certain diseases in aquaculture. *Offiicial Journal of the European Union*, *328*, 14–56. https://doi.org/10.1111/jfd.12323

Evensen, O., Thorud, K. E., & Olsen, Y. A. (1991). A morphological study of the gross and light microscopic lesions of infectious anaemia in Atlantic salmon (Salmo salar). *Research in Veterinary Science*, *51*(2), 215–222. https://doi.org/10.1016/0034-5288(91)90017-I

Everblu Research. (2018). *Seafood Industry Report*. *February*, 21.

Falk, K. (2014). Vaccination against Infectious Salmon Anemia. In *Fish Vaccination* (pp. 313–320). https://doi.org/doi:10.1002/9781118806913.ch26

Falk, K., Aspehaug, V., Vlasak, R., & Endresen, C. (2004). Identification and characterization of viral structural proteins of infectious salmon anemia virus. *Journal of Virology*, *78*(6), 3063–3071.

Falk, K., Namork, E., Rimstad, E., Mjaaland, S., & Dannevig, B. H. (1997). Characterization of infectious salmon anemia virus, an orthomyxo-like virus isolated from Atlantic salmon (Salmo salar L.). *Journal of Virology*, *71*(12), 9016–9023.

Galea, S., Street, S., & Dunlevie, J. (2018). *Macquarie Harbour salmon: 1.35 million fish deaths prompt call to "empty" waterway of farms*. ABC News. https://www.abc.net.au/news/2018-05-29/salmon-deaths-in-macquarie-harbour-top-one-million-epa-says/9810720

García-Rosado, E., Markussen, T., Kileng, Ø., Baekkevold, E. S., Robertsen, B., Mjaaland, S., & Rimstad, E. (2008). Molecular and functional characterization of two infectious

salmon anaemia virus (ISAV) proteins with type I interferon antagonizing activity. *Virus Research*, *133*(2), 228–238. https://doi.org/10.1016/j.virusres.2008.01.008

Genscript. (n.d.). *Real-time PCR (TaqMan) Primer and Probes Design Tool*. https://www.genscript.com/tools/real-time-pcr-taqman-primer-design-tool?src=google&gclid=CjwKCAjw8df2BRA3EiwAvfZWaKVeU58d04xUzWRm8Prz4PsnD31BqhmlV1jX_MrnpAF3ejXQ6M3UpBoCkRsQAvD_BwE

Godoy, M. G., Aedo, A., Kibenge, M. J. T., Groman, D. B., Yason, C. V, Grothusen, H., Lisperguer, A., Calbucura, M., Avendaño, F., & Imilán, M. (2008). First detection, isolation and molecular characterization of infectious salmon anaemia virus associated with clinical disease in farmed Atlantic salmon (Salmo salar) in Chile. *BMC Veterinary Research*, *4*(1), 28.

Godwin, S. ., Beddome, G., Carson, J., Crane, Ms., Douglas, M., Hoad, J., Knowles, G., Michalski, W., Mohr, P., Morrison, R., Peck, G., Shiell, B., Williams, L., Wilson, T., & Moody, N. (2016). *Establishing Viral Diagnostics for salmonid aquaculture in Tasmania*.

Godwin, S. E., Morrison, R. N., Knowles, G., Cornish, M. C., Hayes, D., & Carson, J. (2020). Pilchard orthomyxovirus (POMV). II. Causative agent of salmon orthomyxoviral necrosis, a new disease of farmed Atlantic salmon Salmo salar. *Dis Aquat Organ*. https://doi.org/https://doi.org/10.3354/dao03469

Goić, B., Bustamante, J., Miquel, A., Alvarez, M., Vera, M. I., Valenzuela, P. D. T., & Burzio, L. O. (2008). The nucleoprotein and the viral RNA of infectious salmon anemia virus (ISAV) are localized in the nucleolus of infected cells. *Virology*, *379*(1), 55–63.

Håstein, T. (ED. . (1997). *Workshop on Infectious Salmon Anaemia*. *18*, 92.

Håstein, T., Hill, B. J., & Winton, J. R. (1999). Successful aquatic animal disease emergency programmes. *Revue Scientifique et Technique (International Office of Epizootics)*, *18*(1), 214–227.

Hellebo, A., Vilas, U., Falk, K., & Vlasak, R. (2004). Infectious Salmon Anemia Virus Specifically Binds to and Hydrolyzes 4-O-Acetylated Sialic Acids. *The Journal of Virology*, *78*(6), 3055. https://doi.org/10.1128/JVI.78.6.3055-3062.2004

Hodneland, K., & Endresen, C. (2006). Sensitive and specific detection of Salmonid alphavirus using real-time PCR (TaqMan ®). *Journal of Virological Methods*, *131*(2),

184–192. https://doi.org/10.1016/j.jviromet.2005.08.012

Hovland, T., Nylund, A., Watanabe, K., & Endresen, C. (1994). Observation of infectious salmon anaemia virus in Atlantic salmon, Salmo salar L. *Journal of Fish Diseases*, *17*(3), 291–296. https://doi.org/10.1111/j.1365-2761.1994.tb00224.x

Huon Aquaculture. (2016). *MANAGING SUSTAINABLE GROWTH HUON AQUACULTURE GROUP LIMITED ANNUAL REPORT 2016*.

Huon Aquaculture. (2017). *Tasmanian Salmonid Industry Discussion Paper*. *August*. https://www.huonaqua.com.au/wp-content/uploads/2017/08/Huon-Aquaculture-Salmonid-Industry-Discussion-Paper.pdf

Huon Aquaculture. (2018). *Huon Aquaculture Group Limited Annual Report 2018*. 116. http://annualreport.huonaqua.com.au/#folio=92

Hyatt, A., Hine, P., Jones, B., Whittington, R., Kearns, C., Wise, T., Crane, M., & Williams, L. (1997). Epizootic mortality in the pilchard Sardinos sagax neopilchardus in Australia and New Zealand in 1995. II. Indentification of a herpesvirus within the gill epithelium. *Dis Aquat Org*, *28*, 17–29.

Ingerslev, H.-C., Pettersen, E. F., Jakobsen, R. A., Petersen, C. B., & Wergeland, H. I. (2006). Expression profiling and validation of reference gene candidates in immune relevant tissues and cells from Atlantic salmon (Salmo salar L.). *Molecular Immunology*, *43*(8), 1194–1201.

International Committee on the Taxonomy of Viruses. (2020). *Orthomyxoviridae*. https://talk.ictvonline.org/ictv-reports/ictv_9th_report/negative-sense-rna-viruses-2011/w/negrna_viruses/209/orthomyxoviridae

Jarp, J., & Karlsen, E. (1997). Infectious salmon anaemia (ISA) risk factors in sea-cultured Atlantic salmon Salmo salar. *Diseases of Aquatic Organisms*, *28*(2), 79–86.

Jones, S. R. M., & Groman, D. B. (2001). Cohabitation Transmission of Infectious Salmon Anemia Virus among Freshwater-Reared Atlantic Salmon. *Journal of Aquatic Animal Health*, *13*(4), 340–346. https://doi.org/10.1577/1548-8667(2001)013<0340:CTOISA>2.0.CO;2

Kibenge, F. S. B., Gárate, O. N., Johnson, G., Arriagada, R., Kibenge, M. J. T., & Wadowska, D. (2001). Isolation and identification of infectious salmon anaemia virus (ISAV) from Coho salmon in Chile. *Diseases of Aquatic Organisms*, *45*(1), 9–18.

Kibenge, F. S. B., Godoy, M. G., Fast, M., Workenhe, S., & Kibenge, M. J. T. (2012). Countermeasures against viral diseases of farmed fish. *Antiviral Research*, *95*(3), 257–281.

Kibenge, F. S. B., Xu, H., Kibenge, M. J. T., Qian, B., & Joseph, T. (2007). Characterization of gene expression on genomic segment 7 of infectious salmon anaemia virus. *Virology Journal*, *4*(1), 34.

Koren, C., & Nylund, A. (1997). Morphology and morphogenesis of infectious salmon anaemia virus replicating in the endothelium of Atlantic salmon Salmo salar. *Diseases of Aquatic Organisms*, *29*(2), 99–109. https://www.int-res.com/abstracts/dao/v29/n2/p99-109/

Kristiansen, M., Frøystad, M. K., Rishovd, A. L., & Gjøen, T. (2002). Characterization of the receptor-destroying enzyme activity from infectious salmon anaemia virus. *Journal of General Virology*, *83*(11), 2693–2697.

Krossøy, B., Devold, M., Sanders, L., Knappskog, P. M., Aspehaug, V., Falk, K., Nylund, A., Koumans, S., Endresen, C., & Biering, E. (2001a). Cloning and identification of the infectious salmon anaemia virus haemagglutinin. *Journal of General Virology*, *82*.

Krossøy, B., Hordvik, I., Nilsen, F., Nylund, A., & Endresen, C. (1999). The Putative Polymerase Sequence of Infectious Salmon Anemia Virus Suggests a New Genus within theOrthomyxoviridae. *Journal of Virology*, *73*(3), 2136–2142.

Krossøy, B., Nilsen, F., Falk, K., Endresen, C., & Nylund, A. (2001b). Phylogenetic analysis of infectious salmon anaemia virus isolates from Norway, Canada and Scotland. *Diseases of Aquatic Organisms*, *44*(1), 1–6.

Lalkhen, A. G., & McCluskey, A. (2008). Clinical tests: sensitivity and specificity. *Continuing Education in Anaesthesia Critical Care & Pain*, *8*(6), 221–223. https://doi.org/10.1093/bjaceaccp/mkn041

Lamb, R. A., & Krug, R. M. (1996). *Orthomyxoviridae: The viruses and their replication* (D. M. Knipe, P. M. Howley, & B. N. Fields (Eds.)). Lippincott-Raven Press .

Lamb, R., & Krug, R. (2001). Orthomyxoviridae: The Viruses and Their Replication. *Fields Virology. Volume 1*, *1*.

Leahy, M. B., Dessens, J. T., Weber, F., Kochs, G., & Nuttall, P. A. (1997). The fourth genus in the Orthomyxoviridae: sequence analyses of two Thogoto virus polymerase proteins

and comparison with influenza viruses. *Virus Research*, *50*(2), 215–224.

Li, C., Greiner-Tollersrud, L., & Robertsen, B. (2016). Infectious salmon anemia virus segment 7 ORF1 and segment 8 ORF2 proteins inhibit IRF mediated activation of the Atlantic salmon IFNa1 promoter. *Fish and Shellfish Immunology*, *52*, 258–262. https://doi.org/10.1016/j.fsi.2016.03.038

Life Technologies Corporation. (2014). TaqMan® Universal PCR Master Mix User Guide (Pub. no. 4304449 Rev. E). *Manual*, *4304449*. https://assets.thermofisher.com/TFS-Assets/LSG/manuals/4304449_TaqManPCRMM_UG.pdf

Lin, D. A., Roychoudhury, S., Palese, P., Clay, W. C., & Fuller, F. J. (1991). Evolutionary relatedness of the predicted gene product of RNA segment 2 of the tick-borne Dhori virus and the PB1 polymerase gene of influenza viruses. *Virology*, *182*(1), 1–7.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods*, *25*(4), 402–408. https://doi.org/https://doi.org/10.1006/meth.2001.1262

Llewellyn, L. C. (2015). The Atlantic Salmon Salmo salar in Australia. *Australian Zoologist*, *37*(4), 541–545. https://doi.org/10.7882/AZ.2015.006

Lovely, J. E., Dannevig, B. H., Falk, K., Hutchin, L., MacKinnon, A. M., Melville, K. J., Rimstad, E., & Griffiths, S. G. (1999). First identification of infectious salmon anaemia virus in North America with haemorrhagic kidney syndrome. *Diseases of Aquatic Organisms*, *35*(2), 145–148.

Lyngøy, C. (2003). Infectious salmon anaemia in Norway and the Faeroe Islands: An industrial approach. *International Response to Infectious Salmon Anaemia: Prevention, Control, and Eradication: Proceedings of a Symposium. Tech Bull 1902*, 97–109.

Lyngstad, T., Kristoffersen, A., Hjortaas, M., Devold, M., Aspehaug, V., Larssen, R., & Jansen, P. (2012). Low virulent infectious salmon anaemia virus (ISAV-HPR0) is prevalent and geographically structured in Norwegian salmon farming. *Diseases of Aquatic Organisms*, *101*(3), 197–206. http://www.int-res.com/abstracts/dao/v101/n3/p197-206

Maclachlan, N. J., & Dubovi, E. J. (2010). *Fenner's Veterinary Virology*. Elsevier Science & Technology. http://ebookcentral.proquest.com/lib/bergen-ebooks/detail.action?docID=662114

Mcbeath, A. J. A., Collet, B., Paley, R., Duraffour, S., Aspehaug, V., Biering, E., Secombes, C. J., & Snow, M. (2006). Identification of an interferon antagonist protein encoded by segment 7 of infectious salmon anaemia virus. *Virus Research*, *115*(2), 176–184. https://doi.org/10.1016/j.virusres.2005.08.005

McCauley, J., Hongo, S., Kaverin, N., Kochs, G., Lamb, R., Matrosovic, M., Peres, D., Palese, P., Presti, R., Rimestad, E., & Smith, G. (2012). *Family - Orthomyxoviridae* (A. M. Q. King, M. J. Adams, E. B. Carstens, & E. J. B. T.-V. T. Lefkowitz (Eds.); pp. 749–761). Elsevier. https://doi.org/https://doi.org/10.1016/B978-0-12-384684-6.00061-6

McGeoch, D., Fellner, P., & Newton, C. (1976). Influenza virus genome consists of eight distinct RNA species. *Proceedings of the National Academy of Sciences of the United States of America*, *73*(9), 3045–3049. https://doi.org/10.1073/pnas.73.9.3045

Mikalsen, A. B., Teig, A., Helleman, A. L., Mjaaland, S., & Rimstad, E. (2001). Detection of infectious salmon anaemia virus (ISAV) by RT-PCR after cohabitant exposure in Atlantic salmon Salmo salar. *Diseases of Aquatic Organisms*, *47*(3), 175.

Mjaaland, S., Rimstad, E., Falk, K., & Dannevig, B. H. (1997). Genomic characterization of the virus causing infectious salmon anemia in Atlantic salmon (Salmo salar L.): an orthomyxo-like virus in a teleost. *The Journal of Virology*, *71*(10), 7681.

Mobsby, D and Koduah, A. (2017). Australian fisheries and aquaculture statistics 2016. In *Fisheries Research and Development Corporation project 2014/245* (Issue December).

Mobsby, D. (2018). *Australian fisheries and aquaculture statistics 2017* (Fisheries, Issue December). Fisheries Research and Development Corporation project 2014/245. https://doi.org/https://doi.org/10.25814/5c07b19d3fec4

Mobsby, D., Steven, A. H., & Curtotti, R. (2020). *Australian fisheries and aquaculture outlook 2020* (Issue March). https://doi.org/https://doi.org/10.25814/5e4377eb3eea2

Mohr, P., Crane, Ms., Hoad, J., Williams, L., Cummins, D., Neave, M., Shiell, B., Beddome, G., Michalski, W., Peck, G., Samsing, F., Wynne, J., Crameri, S., Hyatt, A., & Moody, N. (2020). Pilchard orthomyxovirus (POMV). I. Characterisation of an emerging virus isolated from pilchards Sardinops saga and Atlantic salmon Salmo salar. *Dis Aquat Organ*, *139*, 35–50. https://doi.org/https://doi.org/10.3354/dao03470

Morrison, R. N., Carson, J., & Knowles, G. (2013). *Assessment of orthomyxo- like virus pathogenicity in Atlantic salmon*.

Mosby, D. (2018). *Australian fisheries and quaculture statistics 2017*. https://doi.org/hhtps//doi.org/10.25814/5c07b19d3fec4

Müller, A., Markussen, T., Drabløs, F., Gjøen, T., Jørgensen, T. Ø., Solem, S. T., & Mjaaland, S. (2010). Structural and functional analysis of the hemagglutinin-esterase of infectious salmon anaemia virus. *Virus Research*, *151*(2), 131–141.

Mullins, J. E., Groman, D. B., & Wadowska, D. (1998). Infectious salmon anaemia in salt water Atlantic salmon (Salmo salar L.) in New Brunswick, Canada. *Bulletin of the European Association of Fish Pathologists*, *18*(4), 110–114.

Munday, B. L., Foster, C. K., Roubal, F. R., & Lester, R. J. G. (1990). PARAMOEBIC GILL INFECTION AND ASSOCIATED PATHOLOGY OF ATLANTIC SALMON, SALMO SALAR, AND RAINBOW TROUT, SALMO GAIRDNERI, IN TASMANIA. In F. O. Perkins & T. C. Cheng (Eds.), *Pathology in Marine Science* (pp. 215–222). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-550755-4.50026-4

Murray, A. G., Smith, R. J., & Stagg, R. M. (2002). Shipping and the spread of infectious salmon anemia in Scottish aquaculture. *Emerging Infectious Diseases*, *8*(1), 1.

Nayak, D. P., Balogun, R. A., Yamada, H., Zhou, Z. H., & Barman, S. (2009). Influenza virus morphogenesis and budding. *Virus Research*, *143*(2), 147–161.

Norwood, C. (n.d.). *Salmon get ready for their 'flu shots.'* Fisheries Research & Development Corporation. Retrieved March 7, 2019, from https://www.frdc.com.au/media-publications/fish/FISH-Vol-26-4/Salmon-get-ready-for-their-flu-shots

Nuttall, P. A., Morse, M. A., Jones, L. D., & Portela, A. (1995). Adaptation of members of the Orthomyxoviridae family to transmission by ticks. *Molecular Basis of Virus Evolution*, 416–425.

Nylund, A, Alexandersen, S., Rolland, J. B., & Jakobsen, P. (1995a). Infectious Salmon Anemia Virus (ISAV) in Brown Trout. *Journal of Aquatic Animal Health*, *7*(3), 236–240. https://doi.org/10.1577/1548-8667(1995)007<0236:ISAVII>2.3.CO;2

Nylund, A, Hovland, T., Hodneland, K., Nilsen, F., & Lovik, P. (1994). Mechanisms for transmission of infectious salmon anaemia (ISA). *Diseases of Aquatic Organisms*, *19*, 95.

Nylund, A, Hovland, T., Watanabe, K., & Endresen, C. (1995b). Presence of infectious salmon anaemia virus (ISAV) in tissues of Atlantic salmon, Salmo salar L., collected

during three separate outbreaks of the disease. *Journal of Fish Diseases*, *18*(2), 135–145.

Nylund, A, Krossøy, B., Watanabe, K., & Holm, J. A. (1996). Target cells for the ISA virus in Atlantic salmon (Salmo salar L.). *Bulletin of the European Association of Fish Pathologists*, *16*(2), 68–72.

Nylund, A, Plarre, H., Karlsen, M., Fridell, F., Ottem, K., Bratland, A., & Sæther, P. (2007). Transmission of infectious salmon anaemia virus (ISAV) in farmed populations of Atlantic salmon (Salmo salar). *Archives of Virology*, *152*(1), 151–179. https://doi.org/10.1007/s00705-006-0825-9

Nylund, Are, Brattespe, J., Plarre, H., Kambestad, M., & Karlsen, M. (2019). Wild and farmed salmon (Salmo salar) as reservoirs for infectious salmon anaemia virus, and the importance of horizontal- and vertical transmission. *PLOS ONE*, *14*(4), e0215478. https://doi.org/10.1371/journal.pone.0215478

OIE. (2010). Principles and Methods of Validation of Diagnostic Assays for Infectious Diseases. *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, May 2009*, 1–18.

OIE. (2019). *Infection With Hpr-Deleted or Hpr0 Infectious Salmon Anaemia Virus* (pp. 1–16).

Palese, P. (2007). Orthomyxoviridae: the viruses and their replication. *Fields Virology*, 1647–1689.

Parikh, R., Mathai, A., Parikh, S., Chandra Sekhar, G., & Thomas, R. (2008). Understanding and using sensitivity, specificity and predictive values. *Indian Journal of Ophthalmology*, *56*(1), 45–50. https://doi.org/10.4103/0301-4738.37595

Plarre, H., Nylund, A., Karlsen, M., Brevik, Ø., Sæther, P. A., & Vike, S. (2012). Evolution of infectious salmon anaemia virus (ISA virus). *Archives of Virology*, *157*(12), 2309–2326. https://doi.org/10.1007/s00705-012-1438-0

Portela, A., & Digard, P. (2002). The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication. *Journal of General Virology*, *83*(4), 723–734.

Raynard, R. S., Snow, M., & Bruno, D. W. (2001). Experimental infection models and susceptibility of Atlantic salmon Salmo salar to a Scottish isolate of infectious salmon anaemia virus. *Diseases of Aquatic Organisms*, *47*(3), 169–174.

https://doi.org/10.3354/dao047169

Reed, L. J., & Muench, H. (1938). A simple method of estimating fifty per cent endpoints. *American Journal of Epidemiology*, *27*(3), 493–497.

Rimstad, E, Falk, K., Mikalsen, A. B., & Teig, A. (1999). Time course tissue distribution of infectious salmon anaemia virus in experimentally infected Atlantic salmon Salmo salar. *Diseases of Aquatic Organisms*, *36*(2), 107.

Rimstad, Espen, Dale, O. B., Dannevig, B. H., & Falk, K. (2011). Infectious salmon anaemia. *Fish Diseases and Disorders, 3*, 143–165.

Rimstad, Espen, Dale, O. B., Dannevig, B. H., & Knut, F. (2002). Infectious salmon anaemia virus. *APMIS : Acta Pathologica, Microbiologica, et Immunologica Scandinavica*, *110*(4), 273–282. http://www.ncbi.nlm.nih.gov/pubmed/12076262

Ritchie, R. J., Cook, M., Melville, K., Simard, N., Cusack, R., & Griffiths, S. (2001a). Identification of infectious salmon anaemia virus in Atlantic salmon from Nova Scotia (Canada): evidence for functional strain differences. *Diseases of Aquatic Organisms*, *44*(3), 171–178.

Ritchie, R. J., Heppell, J., Cook, M. B., Jones, S., & Griffiths, S. G. (2001b). Identification and characterization of segments 3 and 4 of the ISAV genome. *Virus Genes*, *22*(3), 289–297.

Rodger, H. D., Turnbull, T., Muir, F., Millar, S., & Richards, R. H. (1998). Infectious salmon anaemia (ISA) in the United Kingdom. *Bulletin of the European Association of Fish Pathologists*, *18*(4), 115–116.

Rowley, H. M., Campbell, S. J., Curran, W. L., Turnbull, T., & Bryson, D. G. (1999). Isolation of infectious salmon anaemia virus (ISAV) from Scottish farmed Atlantic salmon, Salmo salar L. *Journal of Fish Diseases*, *22*(6), 483–488.

Samsing, F, Hoad, J., Mohr, P., Dearnley, M., & Wynne, J. W. (2019). Comparative transcriptome analysis of pilchard orthomyxovirus (POMV) and infectious salmon anaemia virus (ISAV). *Fish and Shellfish Immunology*, *91*, 464. https://doi.org/10.1016/j.fsi.2019.04.269

Samsing, Francisca, Rigby, M., Tengesdal, H. K., Taylor, R. S., Farias, D., Morrison, R. N., Godwin, S., Giles, C., English, C., Chong, J. R., & Wynne, J. W. (n.d.). *Transmission and infection dynamics of a new orthomyxovirus in Atlantic salmon (Salmo salar)*.

Sandvik, T., Rimstad, E., & Mjaaland, S. (2000). The viral RNA 3′- and 5′-end structure and mRNA transcription of infectious salmon anaemia virus resemble those of influenza viruses. *Archives of Virology*, *145*(8), 1659–1669. https://doi.org/10.1007/s007050070082

Skjold, P. (2014). *Survival of Salmonid alphavirus in seawater under different physical conditions* (Issue August). University of Bergen.

Snow, M, McKay, P., McBeath, A. J. A., Black, J., Doig, F., Kerr, R., Cunningham, C. O., Nylund, A., & Devold, M. (2006). Development, application and validation of a Taqman real-time RT-PCR assay for the detection of infectious salmon anaemia virus (ISAV) in Atlantic salmon (Salmo salar). *Developmental Biology*, *126*(New Diagnostic Technology: Application in Animal Health and Biologics Controls), 133–145.

Snow, Mike, Ritchie, R., Arnaud, O., Villoing, S., Aspehaug, V., & Fraser, C. (2003). Isolation and characterisation of segment 1 of the infectious salmon anaemia virus genome. *Virus Research*, *92*, 99–105. https://doi.org/10.1016/S0168-1702(02)00322-2

Sommerset, I., Krossøy, B., Biering, E., & Frost, P. (2005). Vaccines for fish in aquaculture. *Expert Review of Vaccines*, *4*(1), 89–101. https://doi.org/10.1586/14760584.4.1.89

Australian fisheries and aquaculture statistics 2018, Fisheries Research and Development Corporation project 2014/245 160 (2020).

Subbarao, K., Klimov, A., Katz, J., Regnery, H., Lim, W., Hall, H., Perdue, M., Swayne, D., Bender, C., Huang, J., Hemphill, M., Rowe, T., Shaw, M., Xu, X., Fukuda, K., & Cox, N. (1998). Characterization of an Avian Influenza A (H5N1) Virus Isolated from a Child with a Fatal Respiratory Illness. *Science*, *279*(5349), 393–396. http://www.jstor.org/stable/2894575

Svingerud, T., Holand, J. K., & Robertsen, B. (2013). Infectious salmon anemia virus (ISAV) replication is transiently inhibited by Atlantic salmon type I interferon in cell culture. *Virus Research*, *177*(2), 163–170. https://doi.org/10.1016/j.virusres.2013.08.004

Tassal. (2013). *Annual report 2013*.

Tassal. (2018). *Annual Report 2018*. https://issuu.com/tassal/docs/tassal_annual_report_2018

Taylor, R. S., Kube, P. D., Muller, W. J., & Elliott, N. G. (2009). Genetic variation of gross gill pathology and survival of Atlantic salmon (Salmo salar L.) during natural amoebic gill disease challenge. *Aquaculture*, *294*(3), 172–179.

https://doi.org/https://doi.org/10.1016/j.aquaculture.2009.06.007

Thorud, K., & Djupvik, H. O. (1988). Infectious anaemia in Atlantic salmon (Salmo salar L.). *Bull Eur Assoc Fish Pathol*, *8*(5), 109–111.

Totland, G. K., Hjeltnes, B. ., & Flood, P. . (1996). Transmission of infectious salmon anaemia (ISA) through natural secretions and excretions from infected smelts of Atlantic salmon Salmo salar during their pre- symptomatic phase. *Diseases of Aquatic Organisms*, *26*, 25–31.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B., Remm, M., & Rozen, S. (2012). Primer3--new capabilities and interfaces. *Nucleic Acids Res.*

Vågsholm, I., Djupvik, H. O., Willumsen, F. V., Tveit, A. M., & Tangen, K. (1994). Infectious salmon anaemia (ISA) epidemiology in Norway. *Preventive Veterinary Medicine*, *19*(3–4), 277–290.

Veterinærinstituttet. (2020). Fiskehelserapporten. In *Veterinærinstituttet 2019* (Issue 1).

Vike, S. (2014). Infectious salmon anaemia in Atlantic salmon, Salmo salar L. in Chile. In *Dissertation* (Vol. 5287). Univeristy of Bergen.

Vike, S., Oelckers, K., Duesund, H., Erga, S. R., Gonzalez, J., Hamre, B., Frette, Ø., & Nylund, A. (2014). Infectious salmon anemia (ISA) virus: infectivity in seawater under different physical conditions. *Journal of Aquatic Animal Health*, *26*(1), 33–42.

Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M., & Kawaoka, Y. (1992). Evolution and ecology of influenza A viruses. *Microbiology and Molecular Biology Reviews*, *56*(1), 152–179.

Weli, S. C., Aamelfot, M., Dale, O. B., Koppang, E. O., & Falk, K. (2013). Infectious salmon anaemia virus infection of Atlantic salmon gill epithelial cells. *Infectious Salmon Anaemia Virus Infection of Atlantic Salmon Gill Epithelial Cells*, *10*(1), 1999.

Whittington, R. J., Crockford, M., Jordan, D., & Jones, B. (2008). Herpesvirus that caused epizootic mortality in 1995 and 1998 in pilchard, Sardinops sagax neopilchardus (Steindachner), in Australia is now endemic. *Journal of Fish Diseases*, *31*(2), 97–105. https://doi.org/10.1111/j.1365-2761.2007.00869.x

Whittington, R., Jones, B., Hine, P., & Hyatt, A. (1997). Epizootic mortality in the pilchard (Sardinops sagax neopilchardus) in Australia and New Zealand in 1995. I. Pathology and

epizootiology. *Dis Aquat Org*, *28*, 1–15.

Wolf, A., Hodneland, K., Frost, P., Braaen, S., & Rimstad, E. (2013). hemagglutinin-esterase-expressing salmonid alphavirus replicon protects Atlantic salmon (Salmo salar) against infectious salmon anemia (ISA). *Hemagglutinin-Esterase-Expressing Salmonid Alphavirus Replicon Protects Atlantic Salmon (Salmo Salar) against Infectious Salmon Anemia (ISA)*, *31*(4), 661–669.

Workenhe, S. T., Wadowska, D. W., Wright, G. M., Kibenge, M. J. T., & Kibenge, F. S. B. (2007). Demonstration of infectious salmon anaemia virus (ISAV) endocytosis in erythrocytes of Atlantic salmon. *Virology Journal*, *4*(1), 13.

Wu, W. W. H., Sun, B. Y.-H., & Panté, N. (2007). Nuclear import of influenza A viral ribonucleoprotein complexes is mediated by two nuclear localization sequences on viral nucleoprotein. *Virology Journal*, *4*(1), 49. https://doi.org/10.1186/1743-422X-4-49

Yamashita, M., Krystal, M., & Palese, P. (1989). Comparison of the three large polymerase proteins of influenza A, B, and C viruses. *Virology*, *171*(2), 458–466.

Appendices

Appendix 1 – Agpath reduced reaction experiment

Table 9. Testing reduced Agpath reactions. Comparable Ct-values were generated for different reaction volumes, template volumes and plates when tested on a POMV positive head kidney sample. Rxn is reactions and SD standard diviation.

Plate	rxn µL	Template µL	Sample	Assay	Ct	Ct mean	Ct SD
1	25	2	Kidney 67	POMV seg 5	25.42 25.17 25.37	25.32	0.13
2	12.5	1	Kidney 67	POMV seg 5	25.41 25.32	25.36	0.06
2	10	0.7	Kidney 67	POMV seg 5	25.38 25.37	25.37	0.01
3	10	1	Kidney 67	POMV seg 5	25.10 25.06	25.08	0.03
3	10	2	Kidney 67	POMV seg 5	24.08 23.92	24.00	0.11

Similar Ct-values were generated for full and reduced reactions of Agpath with low standard deviations. A template volume of 1 µL in a 10 µL reaction was tested, as it is an easier volume to pipette, and showed comparable Ct-values to the Ct-values generated when added 0.7 µL in a 10 µL reaction. By adding 2 µL template to the 10 µL reaction a Ct-value with a digit less than the Ct-value for 1 µL in a 10 µL was generated, supporting that a reduced reaction of 10 µL seems to generate accurate values. Similar Cts were also shown across different plates (Table 1.)

Appendix 2 – Alignments of POMV and ISAV isolates

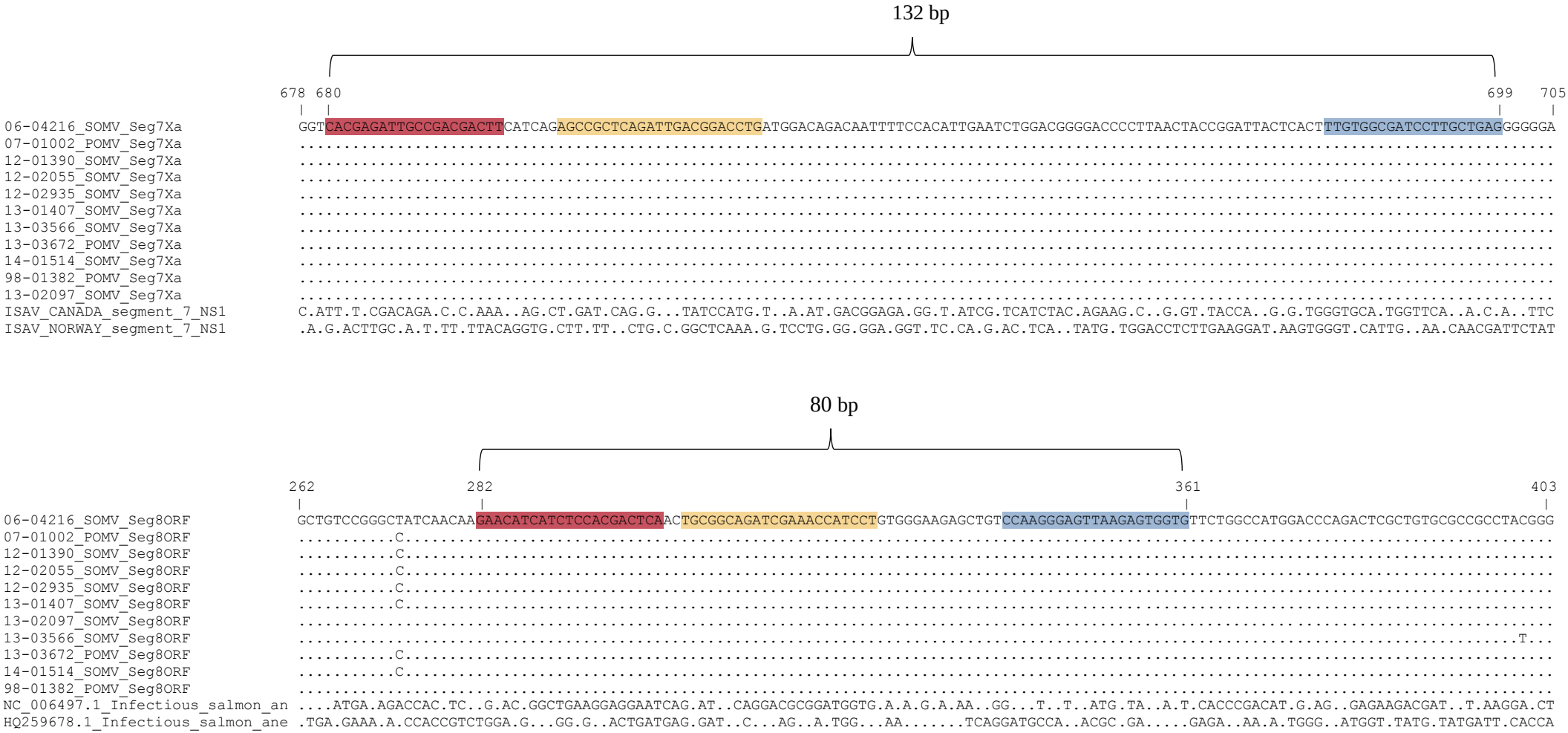


Figure 16. Alignments in Mega 7 involving the entire ORF (only a part of it shown in this figure) of all the 11 available POMV isolates of the respective segment, and the corresponding sequences of European and North American ISAV isolates. The symbol . indicates base pairs identical to the segment 7 and 8 sequence. Nucleotides marked in red indicates forward primer, yellow indicates probe and blue indicates reverse primer. Amplicons length is here shown to be 132 and 80 base pairs (bp) for segment 7 and 8 respectively.

Appendix 3 – Figures Identification of amplicons

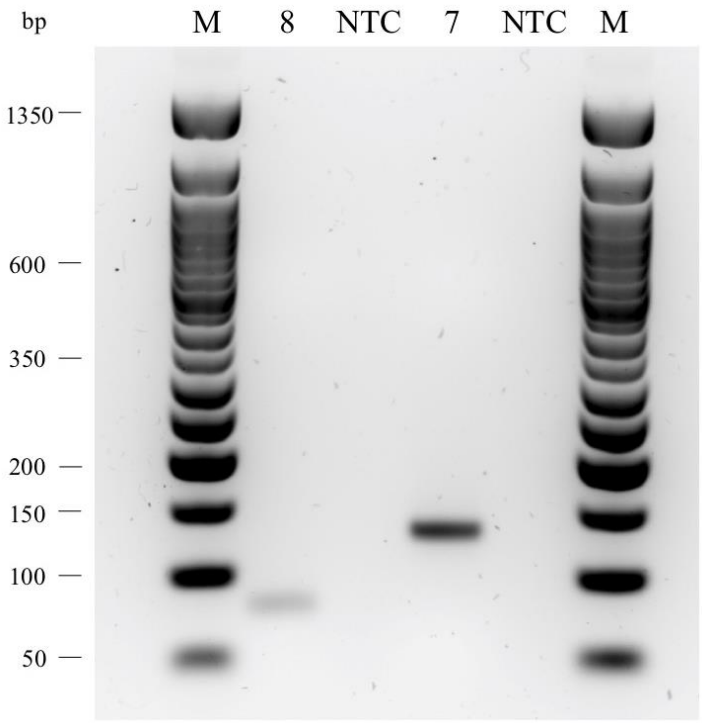


Figure 17. cDNA from a positive POMV fish amplified by endpoint PCR using the newly designed primers for segment 7 and 8, visualized by 3% agarose gel. The positioning of the bands matched the theoretically assessed length of 132 and 80 base pairs (bp) for segment 7 and 8 respectively. The M is DNA-ladder (50 bp), NTC is non-template control, 7 is segment 7 and 8 is segment 8.

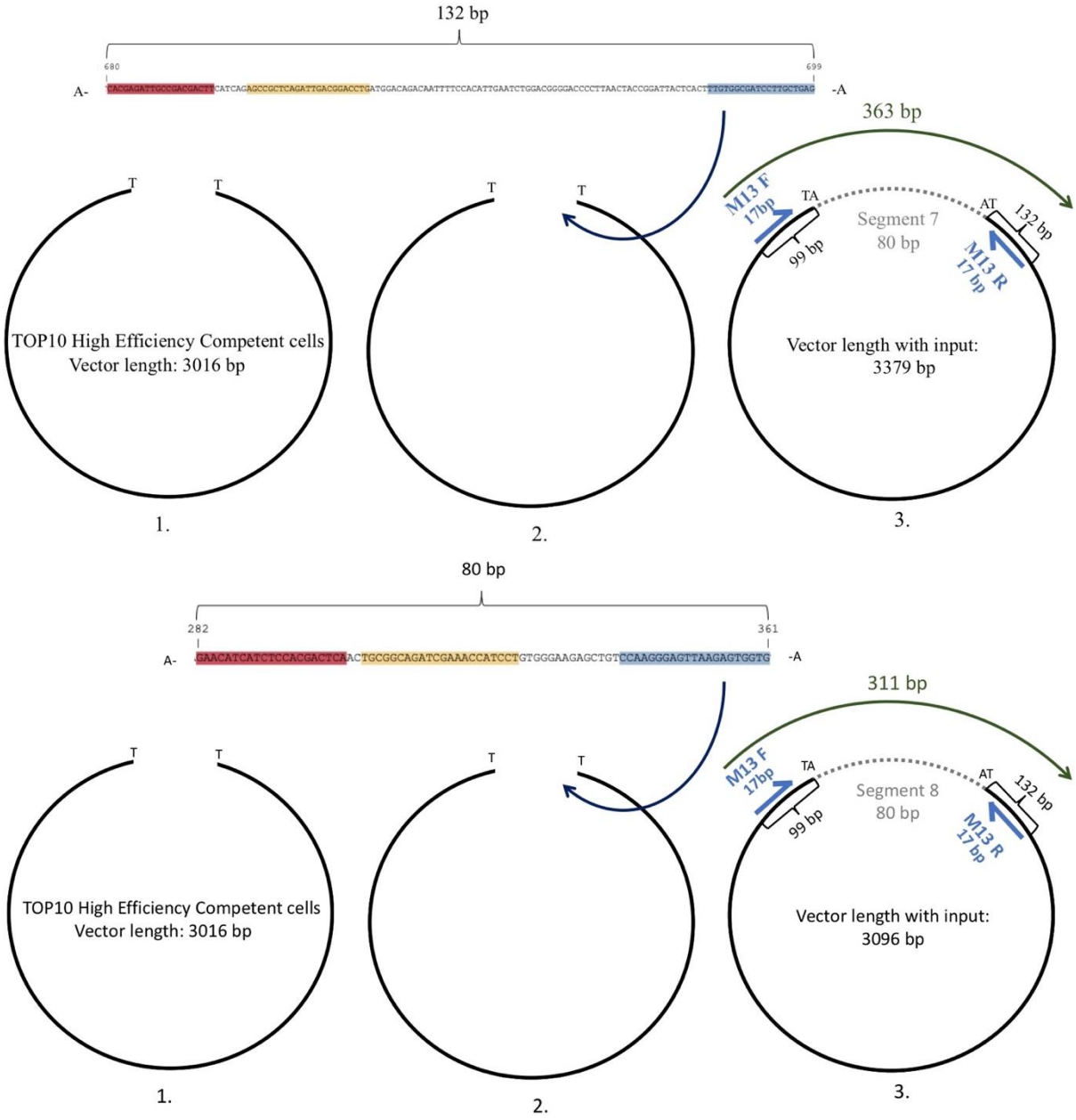


Figure 18. Illustration of the pGEM-T easy vector ligation of cDNA of amplicon diverted from segment 7 (upper figure) and 8 (lower figure) assays. pGEM-T vector ligation is based on TA-cloning, illustrated here in three steps: a vector (plasmid) with T-ends (thyamine) (1) can bind and insert DNA fragments with A-ends (adenine) (2). A-ends has been added to the cDNA during amplification of the respective segment and can be inserted into the plasmid (3). If successful, the vector length will be 3379 and 3096 base pairs (bp) for segment 7 and 8 respectably. Specific primers called M13 forward (F) and reverse primer (R) for the vector can further be used to confirm successful insertion. As illustrated, the amplicon is then expected to be 363 and 311 bp in length for segment 7 and 8 respectively.

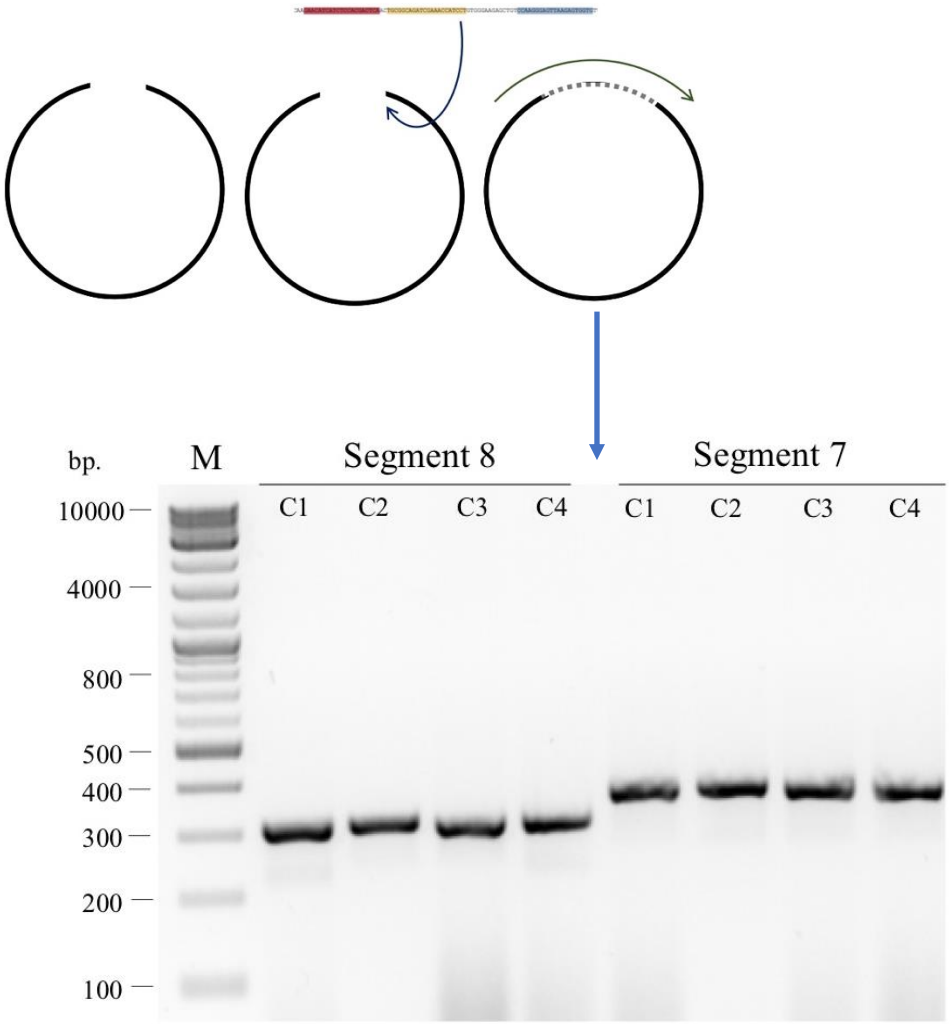
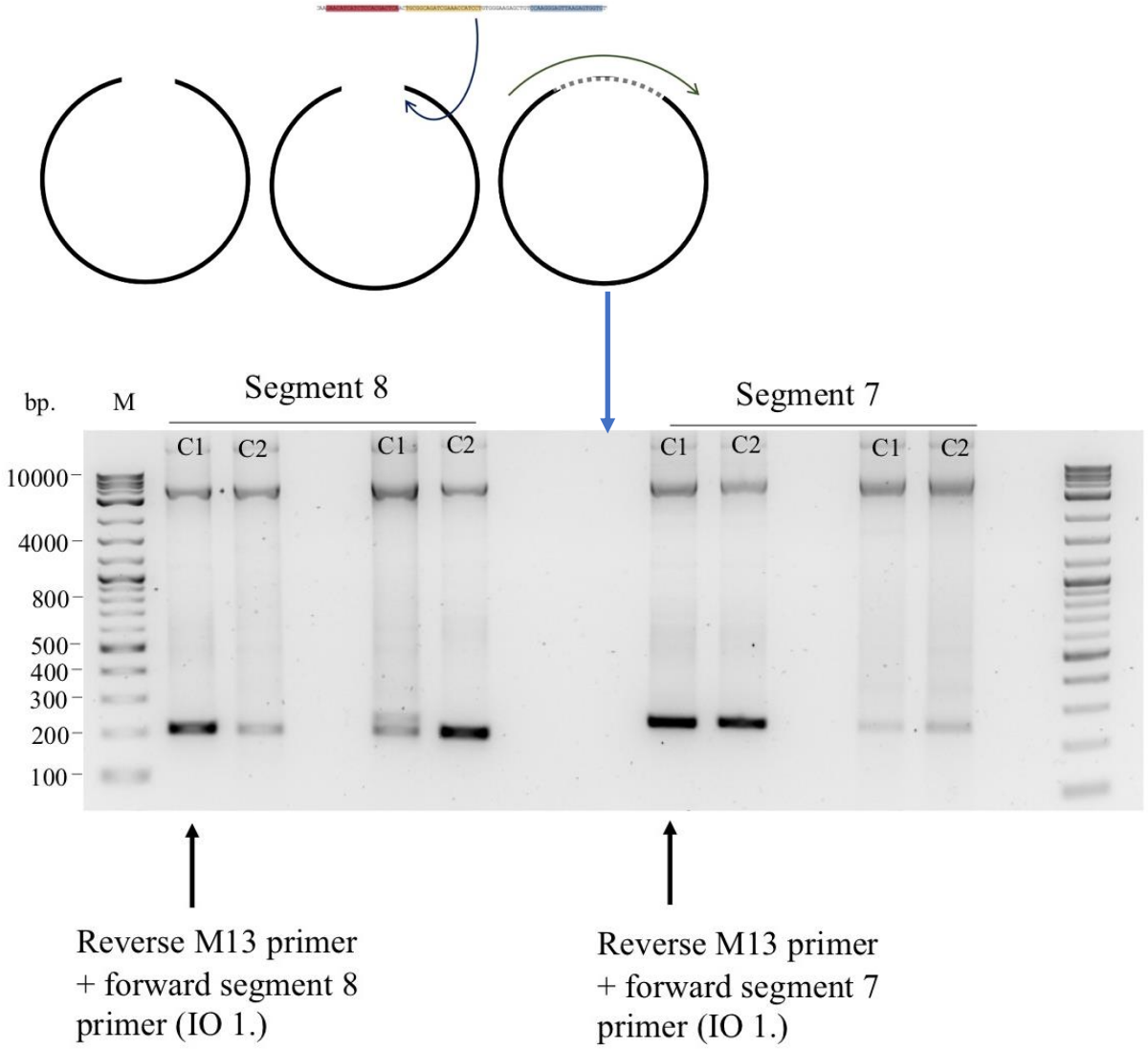


Figure 19. Four colonies of TOP 10 High Efficiency Competent Cells with vectors with segment 7 and 8 inserts were amplified by M13 forward and reverse primers and visualized. The position of the band matches the theoretically assessed amplicon length of 363 and 311 for segment 7 and 8 respectively. M indicates DNA-latter, bp is base pair and C1-C4 indicates colony 1 to 4 of each segment.



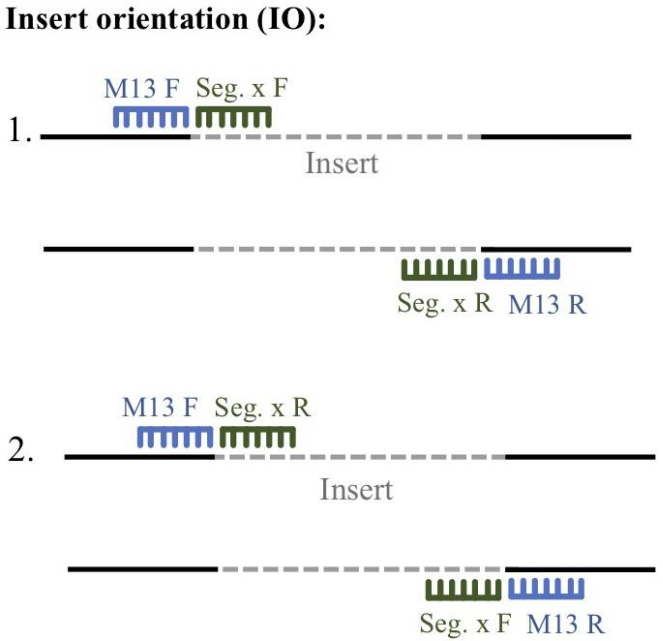
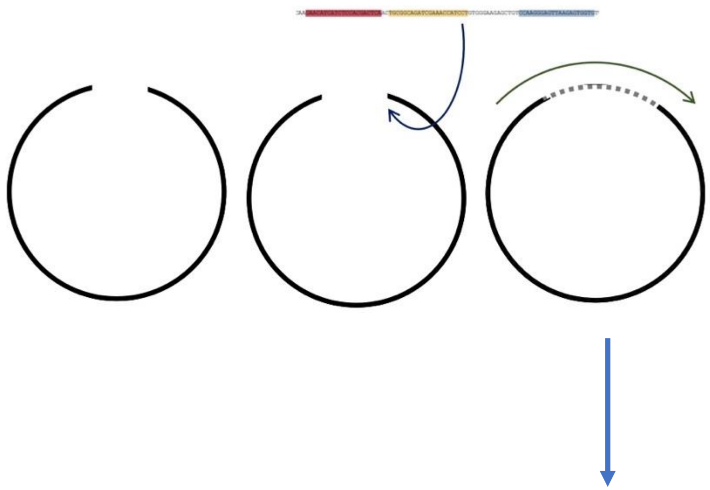


Figure 20. Segment 7 and 8 insert direction in the vector was tested by the use of two different mastermixes for each segment: 1) M13 reverse primer and segment specific forward primer and 2) M13 reverse primer and segment specific forward primer. The PCR-products were visualized by gel electrophoresis. The orientation of the insert was determined by the presence of a band for *either* mastermix 1 or 2 by gel electrophoresis for each colony. Colony C1 for both segment 7 and 8 had insert orientation 1 (IO 1.).



```
                         81                                                                                                                                  212
                         |                                                                                                                                   |
S7-sanger_sequencing GATTCACGAGATTGCCGACGACTTCATCAGAGCCGCTCAGATTGACGGACCTGATGGACAGACAATTTTCCACATTGAATCTGGACGGGGACCCCTTAACTACCGGATTACTCACTTTGTGGCGATCCTTGCTGAGAATC
S7_Amplicon          ----....................................................................................................................................----


                          73                                                                              152
                          |                                                                               |
S8-sanger_sequencing CGATTGAACATCATCTCCACGACTCAACTGCGGCAGATCGAAACCATCCTGTGGGAAGAGCTGTCCAAGGGAGTTAAGAGTGGTGAATCA
S8_Amplicon          -----................................................................................-----
```

Figure 21. Colony C1 for TOP 10 High Efficiency Competent Cells with vectors with segment 7 and 8 inserts was sequenced by Sanger sequencing and aligned with theoretical amplicon assessed from MEGA 7. Both sequences aligned perfectly, confirming that the two assays amplifies the desired sequence.

Appendix 4 – Optimization of primer and probe concentrations

Table 10. Optimisation of primer concentrations for RT-qPCR assays designed to detect segment 8 and 7 of pilchard orthomyxovirus (POMV) on plasmids. Results presented as mean Ct value and standard deviation. Values with a ϕ symbol indicate the chosen concentration.

Concentration (nM) of Fwd and Rv primers	**Segment 8**		**Segment 7**	
	Ct mean	Ct SD	Ct mean	Ct SD
200F_200R	22.712	0.133	24.367	0.032
200F_400R	22.547	0.028	24.059	0.056
200F_900R	**22.374ϕ**	**0.040**	**23.768ϕ**	**0.051**
400F_200R	22.792	0.218	24.473	0.127
400F_400R	22.777	0.086	24.214	0.049
400F_900R	22.526	0.051	23.817	0.052
900F_200R	23.129	0.093	24.566	0.090
900F_400R	22.916	0.031	24.363	0.108
900F_900R	22.907	0.099	24.130	0.057

Table 11. Optimisation of probe concentrations for RT-qPCR assays designed to detect segment 8 and 7 of pilchard orthomyxovirus (POMV) on plasmids. Results presented as mean Ct value and standard deviation. Values with a ϕ symbol indicate the chosen concentration.

Concentration (nM) of probe	**Segment 8**		**Segment 7**	
	Ct mean	Ct SD	Ct mean	Ct SD
250	22.424	0.145	**22.525ϕ**	**0.059**
180	22.111	0.029	22.620	0.062
120	**21.974ϕ**	**0.067**	22.991	0.123
60	22.578	0.288	23.852	0.147

Appendix 5 – Standard curves of segment 7 and 8 assay

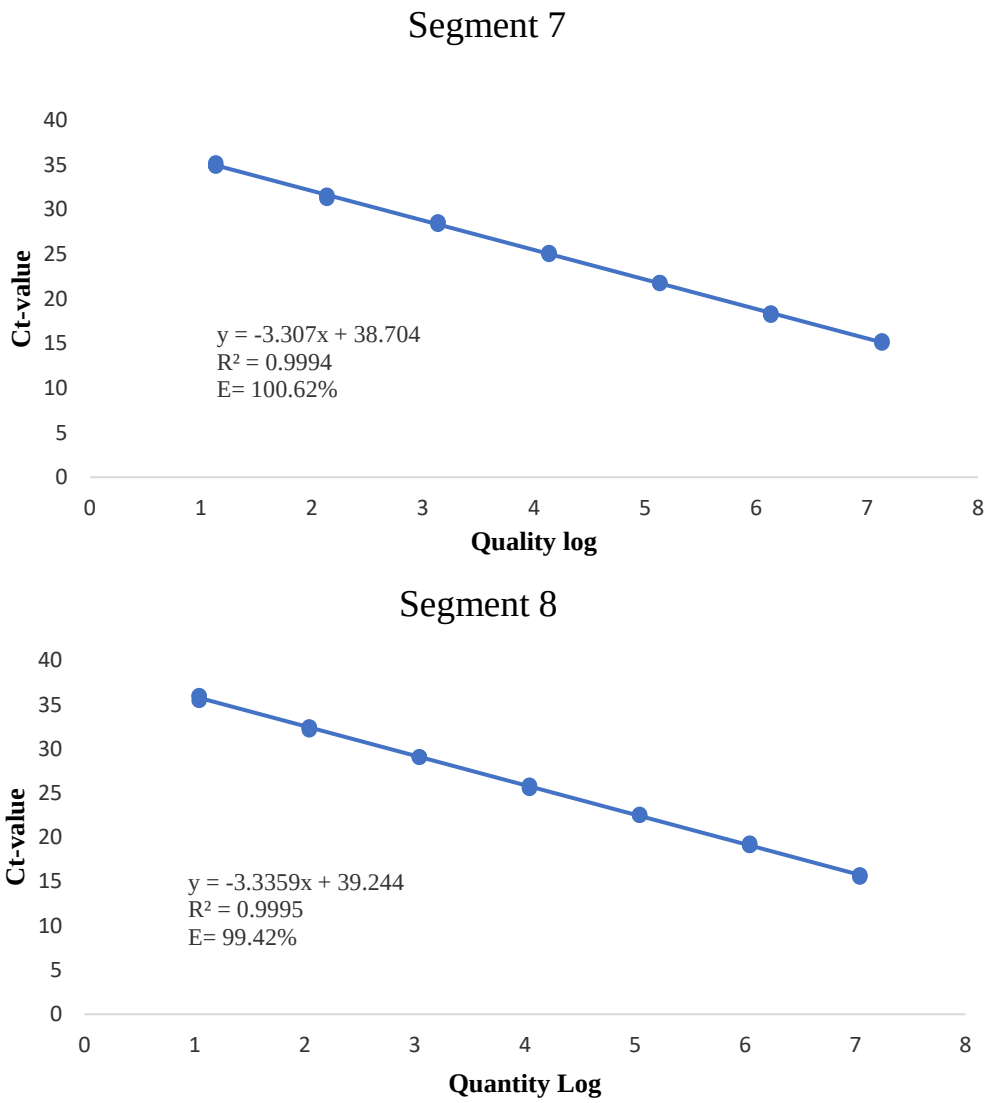


Figure 22. Standard curve involving amplification of 7-fold dilution of plasmid DNA specific to (A) segment 8 and (B) segment 7. E is amplification efficiency.

Appendix 6 - Limit of detection of segment 7 and 8 assay

Table 12. Limit of detection (LOD) of the RT-qPCR assays on plasmids specific to segment 8 and segment 7. The Ct LOD is in bold, which was then converted to its equivalent number of plasmid copies.

Segment 8						
Dilution	Ct 1	Ct 2	Ct 3	Ct 4	Mean	SD
2^0*	35,506	35,944	36,021	-	35,824	0,278
2^-1	36,886	35,130	37,016	35,005	**36,009**	1,090
2^-2	35,972	36,020	Undetermined	Undetermined	35,996	0,034
2^-3	Undetermined	Undetermined	Undetermined	Undetermined		

* from last dilution (dilution 10) in standard curve (~10.95 plasmids copies/ul)

LOD= 5.48 plasmid copies/µl

Segment 7						
Dilution	Ct 1	Ct 2	Ct 3	Ct 4	Mean	SD
2^0*	35,223	34,899	34,859	-	34,99367	0,199613
2^-1	34,209	35,319	36,144	35,977	**35,412**	0,878
2^-2	35,969	36,096	Undetermined	35,188	35,751	0,492
2^-3	35,98	36,135	35,969	Undetermined	36,028	0,093

* from last dilution (dilution 10) in standard curve (~13.60 plasmids copies/ul)

LOD= 6.80 plasmid copies/µl