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Development of a new real-time PCR for the detection of pilchard orthomyxovirus (POMV) in apparently healthy fish

Francisca Samsing [a,*], Hedda K. Tengesdal [a,b], Are Nylund [b], Megan Rigby [a], Kathryn H. Wiltshire [c], Richard N. Morrison [d], Scott Godwin [d], Carla Giles [d], Teresa Wilson [d,e], Peter G. Mohr [f], John Hoad [f], James W. Wynne [a]

[a] CSIRO Agriculture and Food, Aquaculture Program, Hobart, TAS, Australia
[b] University of Bergen, Department of Biological Sciences, Norway
[c] South Australian Research and Development Institute (SARDI) Aquatic Sciences and Marine Innovation Southern Australia, Adelaide, SA, Australia.
[d] DPIPWE Centre for Aquatic Animal Health and Vaccines, Launceston, TAS, Australia
[e] DPIPWE Animal Health Laboratory, Launceston, TAS, Australia
[f] CSIRO Australian Centre for Disease Preparedness (ACDP), East Geelong, VIC, Australia

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ABSTRACT

Pilchard orthomyxovirus (POMV) is a virus of concern to the Atlantic salmon aquaculture industry in Tasmania. First isolated from wild pilchards in southern Australia in 1998, the virus is now a recognised pathogen of farmed Atlantic salmon (*Salmo salar*) in Tasmania. While the current real-time PCR for POMV targets segment 5 of the viral genome, recent viral gene expression data suggests that other segments of the POMV genome presented higher transcription levels and thus may be better candidates for the early detection of the virus. This study aimed to design and begin validating a more sensitive reverse transcriptase real-time PCR (RT-qPCR) assay to detect POMV. Primers and probes were developed targeting two independent viral genes derived from segments 7 and 8, which presented higher transcription levels than segment 5 in both cell culture and infected fish. These were compared with the current POMV RT-qPCR. The POMV segment 8 assay had a higher analytical sensitivity than segment 7, detecting at least 1 plasmid copy μl^{-1}, and was 10-fold more sensitive than both POMV segment 7 and 5 assays when analysing nucleic acid from a positive field sample. Both new assays also had high analytical specificity, detecting the 11 POMV isolates tested (inclusivity testing) and not amplifying nucleic acids from other viruses, including ISAV, a related orthomyxovirus. In the latent class model analysis, the diagnostic sensitivity of the segment 8 and 7 assays were higher than segment 5 in 93% and 92% of simulations, respectively. Seven samples (18.4%), all from subclinical fish infected with POMV, returned a positive result only with the segment 8 assay. Both new assays showed reproducible results when applied to aliquots of the same samples tested in three different laboratories. The new POMV segment 8 assay shows promising results as a surveillance tool for detecting POMV in fish without any symptoms.

1. Introduction

Pilchard orthomyxovirus (POMV) was first isolated in 1998 from healthy wild pilchards (*Sardinops sagax*) in coastal waters of southern Australia (Mohr et al., 2020). Later in 2006, a similar orthomyxo-like virus was isolated from farmed Atlantic salmon (*Salmo salar*) in Tasmania during routine surveillance. No further orthomyxo-like viruses were discovered in farmed Atlantic salmon until 2012, when a serious outbreak causing the mortality of more than 500,000 fish was detected in south-eastern Tasmania (Godwin et al., 2020). Outbreak investigations led to the recovery of an orthomyxo-like virus morphologically similar to that isolated in 1998 from wild pilchards. Subsequent genomic studies demonstrated that both viruses shared high nucleotide identity and thereafter were referred to as POMV (Mohr et al., 2020).

POMV is a primary pathogen of Atlantic salmon (*Salmo salar*), causing a systemic disease known as salmon orthomyxoviral necrosis (SON) in both fresh- and marine-water environments, but natural outbreaks have only been detected in sea-pens (Godwin et al., 2020). POMV

* Corresponding author.
E-mail address: Francisca.samsingpedrals@csiro.au (F. Samsing).

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belongs to the *Orthomyxoviridae* family, which includes serious human and animal pathogens such as influenza A viruses and infectious salmon anaemia virus (ISAV), a notifiable fish disease under the regulations of the World Organization for Animal Health (OIE, 2019b). The International Committee on Taxonomy of Viruses (ICTV), in its 2019 release, recognised seven genera within the *Orthomyxoviridae*, with *Isavirus,* and its single species, ISAV*,* as the only recognised genus that infects salmonids (ICTV, 2019). To date, however, researchers have isolated and characterised two additional orthomyxoviruses that affect salmonids: rainbow trout orthomyxovirus (RbtOV) (Batts et al., 2017) and POMV (Godwin et al., 2020; Mohr et al., 2020). Similar to most orthomyxoviruses, ISAV, RbtOV and POMV contain eight negative-sense, single-stranded RNA segments that encode for at least ten putative proteins. Phylogenetic analysis of the POMV PB1 sequence, one of the most conserved proteins encoded in the genome of orthomyxoviruses, showed that POMV clustered most closely with ISAV and RbtOV. However, there was still a long phylogenetic distance between these viruses, with less than 47% sequence identity at the nucleotide level for PB1 (Mohr et al., 2020). These results provided strong evidence that POMV is the type species of a new genus in the *Orthomyxoviridae* family, tentatively named *Sardinovirus*.

Routine diagnosis and surveillance of POMV are currently undertaken using a reverse transcriptase real-time polymerase chain reaction (RT-qPCR) assay targeting the putative polymerase acidic (PA) open reading frame (ORF) encoded on segment 5 (Mohr et al., 2020). However, recent data profiling viral gene expression levels during experimental infections with POMV (Samsing et al., 2020a, 2020b) have indicated that the transcription rate of other POMV genomic segments are higher than segment 5. Based on these results, the aim of this study was to develop and evaluate the validation characteristics of a more sensitive RT-qPCR assay for the detection of POMV in apparently healthy fish. To this end, primers and probes were developed targeting two independent viral genes (derived from segments 7 and 8, encoding putative proteins S7A and S8A) which presented higher transcription levels *in vitro* (Samsing et al., 2020a) and *in vivo* (Samsing et al., 2020b). These assays were compared with the current RT-qPCR used to detect POMV (Mohr et al., 2020) using samples from a laboratory-based POMV challenge experiment (Samsing et al., 2020c). Improving surveillance through the development of tools that can provide an early diagnosis in apparently healthy or subclinical fish is essential to prevent disease spread and minimise economic losses, especially in marine farming environments (Groner et al., 2016).

2. Materials and methods

To develop and evaluate the validation characteristics of the new POMV assays, the principles and methods established in the Manual of Diagnostic Tests for Aquatic Animals established by the World Organization for Animal Health (OIE, 2019a) were followed.

2.1. Assay development

2.1.1. Primer and probe design

Primer pairs and probes were designed to target the ORFs S7A and S8A, encoded by the first ORF of segments 7 and the only ORF of segment 8 in the POMV genome (Mohr et al., 2020), respectively (Table 1). The entire ORF for each of the 11 isolates of POMV (NCBI: txid2732827) was aligned using MEGA v.7 to identify conserved gene regions for segments 7 and 8. To ensure specificity, matching regions of POMV were further tested *in silico* to be different to ISAV and RbtOV by aligning them with the representative ORFs of segment 7 and 8 of the European ISAV Glesvaer/2/90 strain, North American ISAV strain CCBB and RbtOV Idaho/347/1997 strain. Regions that fulfilled these criteria were inserted into GenScript website design tool (https://www.genscript.com/tools/real-time-pcr-taqman-primer-design-tool) and Primer 3 v.4.1.0 (https://bioinfo.ut.ee/primer3/; Untergasser et al., 2012) for primer pair and probe design according to the guidelines. Primer pairs and probes were ordered from Integrated DNA Technologies (IDT).

The primer and probe binding sites were identical among all of the POMV isolates and contained at least 10 mismatches with aligned ISAV sequences (NCBI accession numbers: HQ259677.1, NC_006498.1, HQ259678.1 and NC_006497.1) and RbtOV sequences (NCBI accession numbers: KX882067.1 and KX882068.1). The endogenous control elongation factor 1 alpha (ELF1α) was used as a reference housekeeping gene due to its stable expression in Atlantic salmon (Ingerslev et al., 2006) using primers and probe designed by Moore et al. (2005) in a parallel single-plex assay. The ELF1α probe targets mRNA specifically by spanning two exons such that it would not amplify contaminating salmon genomic DNA (Snow et al., 2006).

2.1.2. Optimisation of primer and probe concentrations

The optimum probe and primer concentration for a single-plex assay were determined by testing different primer and probe concentrations as follows. The concentrations tested for primers were 900 nM (based on Mohr et al., 2020), 400 nM and 200 nM. For the TaqMan® probes, the concentrations tested were 250 nM (based on Mohr et al., 2020), 120 nM (based on AgPath-ID One-Step recommendations) and 60 nM. All reactions were performed in 96-well plates using a 25 μL volume with RNA template from a strong positive POMV tissue sample, confirmed by the segment 5 RT-qPCR assay (Mohr et al., 2020).

The primer concentrations (forward and reverse) were evaluated first using a matrix of reactions. A range of concentrations for each primer were tested against different partner primer concentrations. The probe concentration was held consistent at 250 nM for all of the initial reactions. The primer concentration combination that produced the lowest average C_T value was further tested against a range of probe concentrations. The optimal assay was identified as the combination of TaqMan® probe and forward- and reverse-primers yielding the lowest average C_T.

The amplicon derived from traditional end-point PCR using each of the newly developed primer sets for segment 7 and 8 of POMV were

Table 1
Reverse transcriptase real-time PCR (RT-qPCR) primers and TaqMan® probes.

Target	Primer/Probe	Sequence (5′-3′)	Reaction conc. (nM)	Amplicon size (bp)	Reference
POMV segment 7	Forward	CACGAGATTGCCGACGACTT	900	132	This study
	Reverse	CTCAGCAAGGATCGCCACAA	900		
	TaqMan® probe	FAM - AGCCGCTCAGATTGACGGACCTG - TAMRA	250		
POMV segment 8	Forward	GAACATCATCTCCACGACTCA	900	80	This study
	Reverse	CACCACTCTTAACTCCCTTGG	900		
	TaqMan® probe	FAM - TGCGGCAGATCGAAACCATCCT - TAMRA	250		
POMV segment 5	Forward	ATCAGAAGGGACGGTGGAAG	900	112	Mohr et al., 2020
	Reverse	CGGTCTCGCAACTCTTGATTG	900		
	TaqMan® probe	FAM -TCAAGCCCAAGGAACCGCAAAAGC - TAMRA	250		
ELF1α	Forward	CCCCTCCAGGACGTTTACAAA	900	57	Moore et al., 2005
	Reverse	CACACGGCCCACAGGTACA	900		
	TaqMan® probe	FAM - ATCGGTGGTATTGGAAC - TAMRA	200		

nucleotide sequenced to confirm the correct target was being amplified (data not provided).

2.1.3. Real-time RT-qPCR

RT-qPCR analyses were run in a QuantStudio™ 5 Real-Time PCR System (ThermoFisher™) according to the standard AgPath-ID One-Step RT-PCR kit (ThermoFisher™) cycling parameters. This consists of 10-min reverse transcription at 45 °C, followed by a 10-minute denaturation at 95 °C, then 40 cycles of 95 °C for 15 s and 60 °C for 45 s. The RT-qPCR reactions were run as standard or reduced reaction volumes. A standard 25 μL reaction included 2 μL template (normalized to 10 ng μL^{-1}) RNA, 12.5 μL 2× RT-PCR buffer, 1 μL 25× RT-PCR enzyme mix, and forward and reverse primers and probe to a final concentration based on optimisation results. A reduced 10 μL reaction included 1 μL template (10 ng μL^{-1}) RNA, 5 μL 2× RT-PCR buffer, 0.4 μL 25× RT-PCR enzyme mix, and forward and reverse primers and probe to a final concentration based on optimisation results. A small experiment was conducted to compare standard and reduced AgPath reactions, showing that both generated comparable C_T values (data not shown). The reduced volume was used with samples from the POMV challenge experiment, all other steps used a standard reaction volume.

The reference housekeeping gene ELF1α was used to normalise C_T values. These calculations were performed according to the double-delta C_T ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001) in Microsoft Excel.

2.2. Assay validation: analytical characteristics

2.2.1. Analytical specificity

Analytical specificity (ASp) of the three POMV RT-qPCR assays was assessed by inclusivity and exclusivity testing. For inclusivity testing, nucleic acids extracted from the eleven POMV isolates derived from pilchards and Atlantic salmon from 1998 to 2014 (Mohr et al., 2020) were tested in duplicate. Samples were extracted using the QIAamp viral RNA mini kit (QIAGEN) as per manufacturer's instructions. The assays were performed using AgPath-ID™ One-Step RT-PCR master mix with a 25 μL total reaction volume containing 2 μL of extracted nucleic acid, final concentrations of 900 nM forward and reverse primers, and 250 nM probe. The assays were run in an Applied Biosystems 7500 Fast Real-Time PCR System using the following thermal cycling conditions; 30-min reverse transcription at 48 °C, followed by a 10-min denaturation at 95 °C, then 45 cycles of 95 °C for 15 s and 60 °C for 60 s. These were the PCR conditions used in the analysis described in section 2.2. Results were analysed using a threshold of ΔRn 0.1.

For exclusivity testing, nucleic acids extracted from representative exotic and enzootic finfish viruses were tested. Infectious salmon anaemia virus (ISAV), a distantly related orthomyxovirus, is a major pathogen of salmonid species that are exotic to Australia. Of the enzootic finfish viruses tested, Tasmanian Atlantic salmon reovirus (TSRV-1) (typical) (Zainathan et al., 2017) and Tasmanian aquabirnavirus (TABV), are significant viral pathogens of farmed salmonid species in Tasmania. The significance of TSRV-2 (atypical TSRV), a genotypic variant of TSRV, in salmonid aquaculture in Tasmania is unknown, but this virus has been isolated intermittently over the last 30 years during routine health surveillance.

2.2.2. Analytical sensitivity

Analytical sensitivity (ASe) of the two POMV RT-qPCR assays was assessed by determining their limit of detection (LOD) using serial 10-fold dilutions of amplicon-specific plasmid DNA as template. Ten-fold dilution series (10^7 to 10^{-4} copies μL^{-1}) were prepared in TE buffer, pH 8.0, containing 50 ng μL^{-1} yeast tRNA. The LOD for the two plasmids was estimated at the dilution where three of three replicates returned C_T values from typical amplification curves. Each dilution point of the standard curve was run in triplicate, and the mean C_T value was plotted against the dilution series. Amplification efficiency was calculated based on the C_T slope method (Efficiency = $[10^{(-1/\text{slope})} - 1] \times 100$) and the linearity was demonstrated with the coefficient of determination (R^2). The testing of amplicon-specific plasmid DNA was an initial attempt to assess (via DNA polymerase activity only) if the newly designed RT-qPCR assays could detect target nucleotides to single copy numbers per reaction as is standard for real-time PCR assays.

Amplicon-specific DNA plasmids were generated as follows. RNA extracted from a strong positive POMV tissue sample (from Samsing et al., 2020c) was transcribed into cDNA using Superscript® IV Reverse Transcriptase with Oligo d(T) primers following manufacturer's instructions (Thermo Fisher Scientific, Waltham, USA). The cDNA (1 μL) was used as template in each standard 50 μL reaction which contained (per reaction) 5 μL 10× Platinum Taq 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 Polymerase High Fidelity (5 U μL^{-1}), 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 s, annealing at 55 °C for 30 s, elongation at 68 °C for 30 s in an Eppendorf Mastercycler EP Gradient S (Hamburg, Germany). PCR-products of desired size were confirmed by performing a 3% agarose gel electrophoresis. PCR-products were then 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) and the resulting plasmid was used to transform using One Shot® TOP10 chemically competent E. coli. (Thermo Fisher Scientific, Waltham, USA). Transformed cells were plated onto LB agar plates containing ampicillin (100 mg L^{-1}) and incubated overnight at 37 °C. Successful ligation and transformation was confirmed by PCR of multiple colonies using M13 vector specific primers. Colonies with inserts were grown in LB broth overnight at 37 °C. Corresponding glycerol (20%) stocks were prepared for archiving and plasmids were purified using PureYieldTM Plasmid Miniprep System (Promega Madison, USA). Plasmid DNA concentrations were measured by Qubit dsDNA BR assay kit (Thermo Fisher Scientific,Waltham, USA). The purified plasmids together with the M13 forward primer were sent to Ramaciotti Centre for Genomics (UNSW Sydney) for Sanger sequencing in one direction to confirm insertion of the amplicon (data not shown).

Analytical sensitivity was also estimated for the POMV segment 5, 7 and 8 assays using nucleic acid extracted from a field sample from a clinical disease event at a commercial Atlantic salmon (*S. salar*) farm in southeast Tasmania. The field sample, consisting of 10% *w/v* clarified tissue homogenate (pooled head-kidney, liver and spleen), was serially 10-fold diluted (10^{-1} to 10^{-9}) in nuclease-free water and tested as described in section 2.2.1.

2.3. Assay validation: diagnostic characteristics

2.3.1. Study population and sample collection

Samples used for assay validation were sourced from the experimental challenge with POMV as described in Samsing et al. (2020c). All animal procedures were approved by the DPIPWE Animal Ethics Committee (AEC) under AEC project number 3/2018–19 according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8th edition, 2013). In brief, Atlantic salmon post-smolts were challenged in seawater via cohabitation with trojan fish (or POMV "shedders"), intraperitoneally (IP)-injected with 200 μL of cell culture supernatant containing POMV at a titre of $10^{8.8}$ $TCID_{50}$ ml^{-1} (tissue culture infective dose 50% per millilitre). Twenty-two trojan fish were randomly allocated into each of the four replicated cohabitation tanks, and these were additionally stocked with 32 cohabitation fish per tank. Nine randomly chosen fish were sampled on day zero from a holding tank prior to injection of trojan fish and used as a pre-challenge control.

For 20 days post-infection (dpi), the tanks were monitored three times daily and moribund fish were immediately removed and euthanised with 100 mg L^{-1} of AQUI-S™, necropsied and sampled. Three apparently healthy fish – subclinical fish not displaying gross clinical

signs of POMV – were sampled from each tank on days 1, 2, 4, 8, 12- and 19- post-infection (dpi) of trojan fish. Samples from subclinical and clinical (moribund) fish were used for testing diagnostic characteristics. These were tissue samples of head-kidney as these presented the highest viral load in the early stages of infection (Samsing et al., 2020c).

2.3.2. RNA extraction

Tissue samples were stored in 1 ml RNAlater® (Invitrogen) at −80 °C before extraction. Total RNA was extracted from tissue samples using the RNeasy Plus Kit (Qiagen). Tissue samples were weighed (~ 20 mg) and placed into Lysing Matrix E 2 mL tubes (MP Biomedicals™) for disruption and homogenisation with RLT-Plus buffer/β-mercaptoethanol (600 μL) in a TissueLyser II (Qiagen) for 2 mins at 20 Hz. The remaining procedures were done as per the manufacturer's instructions. After extraction, total RNA was eluted in 40 μL of RNase-free water. PCR assays were conducted as described in section 2.1.3. RNA quantity and quality were estimated using ultraviolet (UV) absorbance in a NanoDrop® 8000 spectrophotometer (Thermo Scientific).

2.4. Statistical analysis

The diagnostic sensitivity (DSe) and specificity (DSp) of the three assays were estimated using a Bayesian Latent Class Model (LCM) allowing for conditional dependence (covariance) between tests, following Buss et al. (2019) and Caraguel et al. (2019). Code is provided in the supplementary material.

Thirty-eight samples from the POMV challenge experiment described previously were used for the assessment of DSe and DSp, and included naïve or pre-challenge controls ($n = 5$), subclinical or apparently healthy fish from all sampling points ($n = 26$) and moribund fish ($n = 7$). Moribund fish were included in the analysis as a reference or benchmark, because all three assays should detect clinically affected fish.

Prior estimates of DSe and DSp were obtained using the 'betaExpert' function in the R package *prevalence* (v.0.4.0; Devleesschauwer et al., 2015), which calculates the parameters of a beta distribution based on experts opinion. For the LCM, beta priors (28, 7) reflecting 95% confidence that DSe estimates were between 0.7 and 0.99 with an expected mean of 0.8 were used for the three assays. Similarly, beta priors (23.6, 0.5) reflecting 95% confidence that DSp estimates were between 0.9 and 0.99 with an expected mean of 0.98 were used for the three assays. Conditional covariance parameters were given uniform priors based on DSe and DSp with bounds set on minimum and maximum possible values, 0 and 1 respectively (Gardner et al., 2000). Covariances between tests were considered important if their 95% credible intervals (CI) did not cross zero.

Latent class model (LCM) analyses using Markov Chain Monte Carlo (MCMC) simulations were obtained by running the model in JAGS v.4.3.0. Simulations were run using three chains for 50,000 iterations, following 10,000 for burn-in. JAGS was run using the R package interface *R2jags* (Su and Yajima, 2015). Convergence was assessed by Gelman-Rubin statistics and confirmed by visual inspection of trace and density plots generated using the *MCMCvis* package (Youngflesh, 2018).

2.5. Reproducibility and inter-laboratory validation

Reproducibility is the ability of a test to provide consistent results when applied to aliquots of the same samples tested in different laboratories using the same assay (OIE, 2019a). Here, the best performing assay based on analytical and diagnostic characteristics (POMV segment 8 RT-qPCR) was tested in three different laboratories: the DPIPWE Animal Health Laboratory in Launceston (Tasmania, Australia), the CSIRO Aquaculture facility in Hobart (Tasmania, Australia) and the CSIRO Australian Centre for Disease Preparedness (ACDP) in Geelong (Victoria, Australia). From hereafter, collaborating laboratories are referred to as laboratories 1, 2 and 3, respectively.

A panel of known POMV-positive and negative samples ($n = 30$) were prepared by laboratory 1 and tested blindly in all three facilities. POMV-positive samples were prepared from gill homogenates derived from Atlantic salmon which had been previously analysed as part of a separate POMV *in vivo* trial (AEC project number 14/2017–2018) conducted at the DPIPWE. Gill tissue is also currently the tissue of choice for POMV surveillance in Tasmania. These fish were challenged by cohabitation with trojan fish that were directly injected with infectious POMV as described above. The panel included both moribund fish that exhibited clear clinical signs consistent with POMV infection ($n = 12$), and fish that were still surviving at 11 days post challenge and exhibited few or no clinical signs ($n = 8$). The panel also included negative (POMV-free) samples collected from the freshwater phase of Atlantic salmon that had never been exposed to POMV. Each sample comprised gill tissue that had been collected from euthanised fish immediately post-mortem and mixed with virus transport medium (VTM, Hanks balanced salts solution supplemented with 2% (*v*/v) foetal bovine serum, 100 U/mL of penicillin G and 100 μg/mL streptomycin sulphate) at a 1:2 (*w*/*v*) ratio of tissue to medium. The samples were homogenised by bead beating with 1.4 mm ceramic beads (MP Biomedicals) using a Precellys tissue homogeniser (Bertin Instruments, France). The 2 mL tubes containing the beads and tissues were processed for 4 × 15 s cycles of 6000 rpm with 30 s pauses between each cycle. The temperature of the bead beater was maintained at 0–4 °C during homogenisation. The homogenised samples were clarified by centrifugation at 1500 *g* for 60 s, then supernatants stored at −80 °C prior to RNA extraction. Samples were transported in dry ice for testing in laboratories 2 and 3.

3. Results

3.1. Assay design and optimisation

After the design of the RT-qPCR assays for POMV segment 7 and segment 8 (Table 1), the concentration of probe and primers within each reaction were optimised. The lowest C_T values in the optimisation matrix were obtained using 200 nM for both forward primers, 900 nM for reverse primers, and 120 nM for the probe in the segment 8 assay, but with very little variation (~ 1% coefficient of variation) between all concentrations in the matrix (Tables S1 and S2). This demonstrated that the assays were robust to small variations in primer and probe concentrations, and 900 nM for all primer pairs and 250 nM for the probes were selected as per the concentrations used for the POMV segment 5 RT-qPCR (Mohr et al., 2020).

3.2. Analytical characteristics

Results from ASp inclusivity testing (Table 2) demonstrated that the two newly developed POMV assays detect the 11 POMV cultured isolates described by Mohr et al. (2020). These isolates represented archived isolates from both pilchards and Atlantic salmon collected in South Australian and Tasmanian waters from 1998 to 2014. Results from ASp exclusivity testing (Table 3) demonstrate that the two newly developed POMV assays do not amplify nucleic acids extracted from ISAV, a related orthomyxovirus, and the enzootic viruses of salmonids in Tasmania TSRV-1, TSRV-2 (atypical TSRV) and TABV.

The LOD for the POMV segment 7 and 8 assays (Table 4) was 2 and 0.2 plasmid copies μL^{-1}, respectively, when diluted in TE buffer containing 50 ng μL^{-1} yeast tRNA. POMV segment 5 plasmid LOD estimation were determined during previously conducted research (Mohr et al., 2020), and was not repeated during this evaluation.

The LODs determined from POMV nucleic acid present in a field sample (Table 5) indicate that the POMV segment 8 assay is 10-fold more sensitive than both the POMV segment 5 and 7 assays that had equivalent LODs.

Table 2
Analytical specificity (ASp) inclusivity testing.

Isolate ID	Year isolated	Fish species	POMV segment 5 RT-qPCR Mean C_T	POMV segment 7 RT-qPCR Mean C_T	POMV segment 8 RT-qPCR Mean C_T
98–01382	1998	Pilchard	15.27	15.87	15.05
06–04216	2006	Atlantic salmon	16.71	17.23	15.48
07–01002	2007	Pilchard	14.33	14.68	13.88
12–01390	2012	Atlantic salmon	13.15	13.91	12.53
12–02055	2012	Atlantic salmon	12.15	12.97	11.84
12–02935	2012	Atlantic salmon	15.26	15.72	14.43
13–01407	2013	Atlantic salmon	15.33	15.43	14.04
13–02097	2013	Atlantic salmon	15.03	15.30	14.25
13–03566	2013	Atlantic salmon	17.61	17.94	16.74
13–03672	2013	Pilchard	12.95	13.64	12.46
14–01514	2014	Atlantic salmon	15.80	16.27	13.98

Table 3
Analytical Specificity (ASp) exclusivity testing (– = Negative).

Virus	Isolate ID	POMV segment 5 RT-qPCR Mean C_T	POMV segment 7 RT-qPCR Mean C_T	POMV segment 8 RT-qPCR Mean C_T
ISAV	ISAV Glesvaer 2/90	–	–	–
TSRV-1	90–00388	–	–	–
TSRV-2	14–02611	–	–	–
TABV	98–00047	–	–	–
TABV	09–01288	–	–	–

Table 4
Analytical Sensitivity (ASe) measured as limit of detection (LOD) and efficiency of the POMV segment 7 and 8 RT-qPCR assays. The dilution series were prepared in TE buffer, pH 8.0, containing 50 ng μl^{-1} yeast tRNA. Values in bold with a ϕ symbol indicate the LOD. (– = Negative).

Plasmid copies per µL	POMV segment 7 RT-qPCR Mean C_T	POMV segment 8 RT-qPCR Mean C_T
2×10^7	10.02	8.98
2×10^6	14.44	12.88
2×10^5	17.83	16.30
2×10^4	21.35	19.64
2×10^3	24.70	22.93
2×10^2	28.01	26.33
2×10^1	31.34	29.69
2×10^0	**34.09ϕ**	32.70
2×10^{-1}	36.69*	**36.03ϕ**
2×10^{-2}	–	–
2×10^{-3}	–	–
2×10^{-4}	–	–
Efficiency	100%	98.75%
R^2	0.996	0.999

* Denotes dilutions where the mean C_T was calculated from less than 3 replicates.

3.3. Diagnostic characteristics

All three tests agreed on a negative result in 13 samples (34.2%), including all naïve or pre-challenge control fish, and on a positive result in 12 samples (31.5%) (C_T range: 15.7–37.2). (Fig. 1). Seven samples (18.4%), all from subclinical fish, returned a positive result only in the POMV assay targeting segment 8 (C_T range: 30.8–35.4) and five samples (13.2%) were positive to both segment 7 (C_T range: 34.9–36.4) and 8 (C_T range: 29.1–32.3), but negative to segment 5. Results for all three assays are presented in Table S3.

There was no evident lack of MCMC convergence in the LCM analyses based on Gelman-Rubin statistics (≤ 1.01 for all parameters) and visual assessment of trace plots (Fig. S2 and S3). For individual tests, the DSe (mean, 95% CI) was higher for the POMV segment 8 RT-qPCR assay (0.84, 0.73–0.93) and segment 7 (0.83, 0.71–0.93), lower for segment 5 (0.72, 0.58–0.85) with a small overlap in the 95% CIs (Fig. 2). However, the percent of MCMC iterations where the DSe of the segment 8 and 7 tests were higher than segment 5 were 93% and 92% of simulations, respectively. DSp was high and similar for all tests (segment 8: 0.86, 0.70–0.99; segment 7: 0.96, 0.85–0.99; segment 5: 0.98, 0.92–0.99) with large overlaps in CIs. None of the covariances between tests were different to zero (all 95% CIs crossed zero).

Table 5
Analytical Sensitivity (ASe) measured as limit of detection (LOD) of the three real-time RT-qPCR assays (segments 5, 7 and 8) using nucleic acid extracted from a POMV positive field sample. Values in bold with a ϕ symbol indicate the LOD. (– = Negative).

Nucleic acid dilution	POMV Segment 5 RT-qPCR Mean C_T	POMV Segment 7 RT-qPCR Mean C_T	POMV Segment 8 RT-qPCR Mean C_T
10^0	15.65	16.10	14.19
10^{-1}	18.76	19.27	17.32
10^{-2}	21.92	22.51	20.58
10^{-3}	25.58	26.06	23.91
10^{-4}	29.08	29.47	27.54
10^{-5}	32.53	32.85	30.94
10^{-6}	**36.26ϕ**	**35.82ϕ**	34.46
10^{-7}	–	–	**36.77ϕ**
10^{-8}	–	–	–
10^{-9}	–	–	–

3.4. Reproducibility

To evaluate the reproducibility of the POMV segment 8 assay, a panel of known POMV-positive and negative samples ($n = 30$) was prepared by laboratory 1, and blindly tested in all three facilities. All samples returned the same results, in all three laboratories except for one that gave a false positive result in laboratory 2, as it was expected to be negative. All results are presented in Table S4. Agreement between C_T values obtained from each laboratory were determined using Lin's concordance correlation coefficient (CCC) (Lin, 1989, 2000), showing a high correlation between C_T values (C_T value range: 15.6–29.8; CCC range between 0.93 and 0.97) obtained by all three laboratories. Reproducibility results including CCC plots and Black and Altman (1999); Bland and Altman, 1986) are presented in Fig. 3.

Laboratory 3 also tested the panel with all three assays and found with agreement between the three assays for all positive and negative samples, except sample 5 that had the highest Seg 8 Ct of the positives and was only positive with the seg 7 and seg 8 assays (Table S5).

4. Discussion

Recent gene expression data profiled during RNA sequencing experiments with POMV (Samsing et al., 2020a, 2020b) had suggested that other segments of the viral genome could be better targets for the early detection of the virus due to higher expression levels. The original assay described by Mohr et al. (2020) was designed for detection of segment 5, but given the relative expression of viral ORFs encoded by segments 7 and 8, these sequences were candidates to increase the sensitivity of POMV detection. This is particularly important in surveillance of

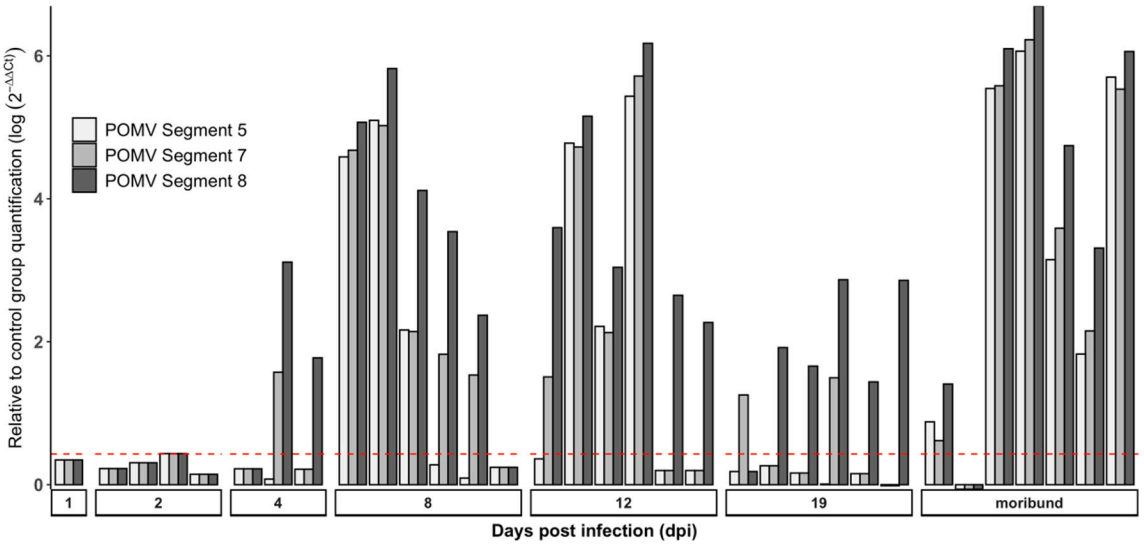

Fig. 1. RT-qPCR results showing individual values for each sample assessed in parallel with the three assays targeting pilchard orthomyxovirus (POMV) segments 5, 7 and 8. All samples were tissue homogenates of head-kidney obtained from Atlantic salmon (*Salmo salar*) post-smolts challenged by cohabitation with POMV-injected fish in seawater in a 20-day trial. Each bar in the plot represents the normalised level of POMV segment 5, 7 or 8 RNA. Tissue samples were collected from subclinical fish (n = 26) on days 1, 2, 4, 8, 12- and 19- post-infection (dpi) and from moribund fish (n = 7), which were animals showing evident clinical signs of POMV (Godwin et al., 2020). All results were normalised against the housekeeping gene elongation factor 1 alpha (ELF1α) using the double-delta C_T method and displayed relative to a fixed cut-off set to C_T 40. Values over the red dotted line represent positive fish. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

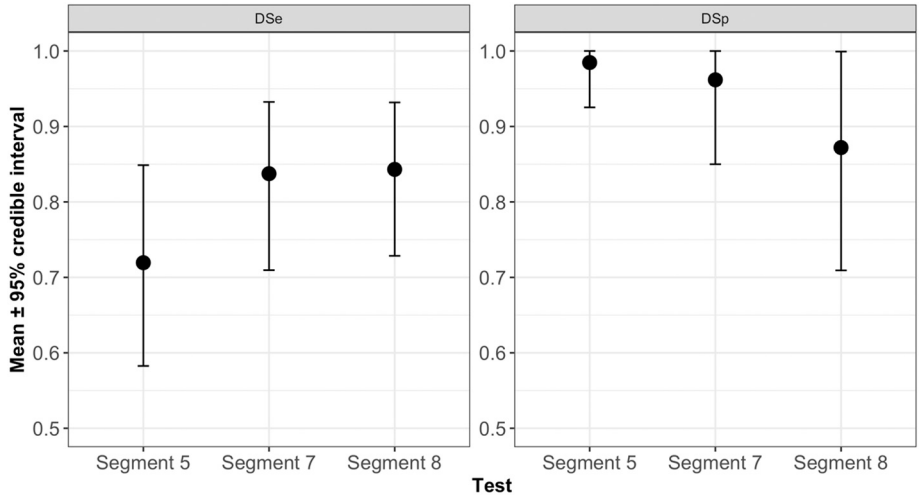

Fig. 2. Latent Class Model calculated mean and 95% credible intervals (CI) for diagnostic sensitivity (DSe) and specificity (DSp) in the RT-qPCR assays targeting segments 5, 7 and 8 of pilchard orthomyxovirus (POMV).

apparently healthy animals. The present study indicated that the segment 8 assay was more sensitive than the POMV segment 7 assay during the initial assessment of analytical sensitivity using dilution series of plasmid DNA. Analytical sensitivity of the POMV segment 8 assay was also higher than both the POMV segment 7 and 5 assays when using nucleic acid extracted from positive field samples. The assays targeting POMV segments 8 and 7 had higher diagnostic sensitivity than the POMV segment 5 assay in experimentally infected fish. Since the assays displayed similar theoretical efficiencies, such a finding is most likely related to the differential expression of viral transcripts during the infection cycle. An increased abundance of segment 8 mRNA has also been described in Atlantic salmon infected with ISAV (Snow et al., 2006). As such, the OIE Manual of Diagnostic Tests for Aquatic Animals (OIE, 2019b) recommends primer-probe sets targeting both ISAV segments 7 and 8 developed by Snow et al. (2006).

In this study, the POMV segment 7 and 8 assays delivered positive results in samples of infected fish that were negative to POMV segment 5. The samples that *only* tested positive for either segment 7 and/or 8 were those that came from sub-clinical (or pre-clinical) fish sampled during the early stages of infection, and those sampled after the peak of infection on day 19 post-exposure. The greater abundance of POMV segments 7 and 8 RNA throughout the infection cycle increases the assay sensitivity in the early stages of infection (pre-viraemia) or post-viraemia, where the assay is probably detecting lingering RNA after the peak of infection (Hodneland and Endresen, 2006).

The development of a new POMV assay with increased sensitivity was initiated after two recent RNA sequencing experiments presenting the transcriptional patterns of POMV genes during host infection. Read mapping to the viral genome revealed that all viral gene segments were expressed within infected host cells (Samsing et al., 2020a) and tissues (Samsing et al., 2020b). However, different patterns were observed for different viral segments, with segments coding for the RNA polymerase

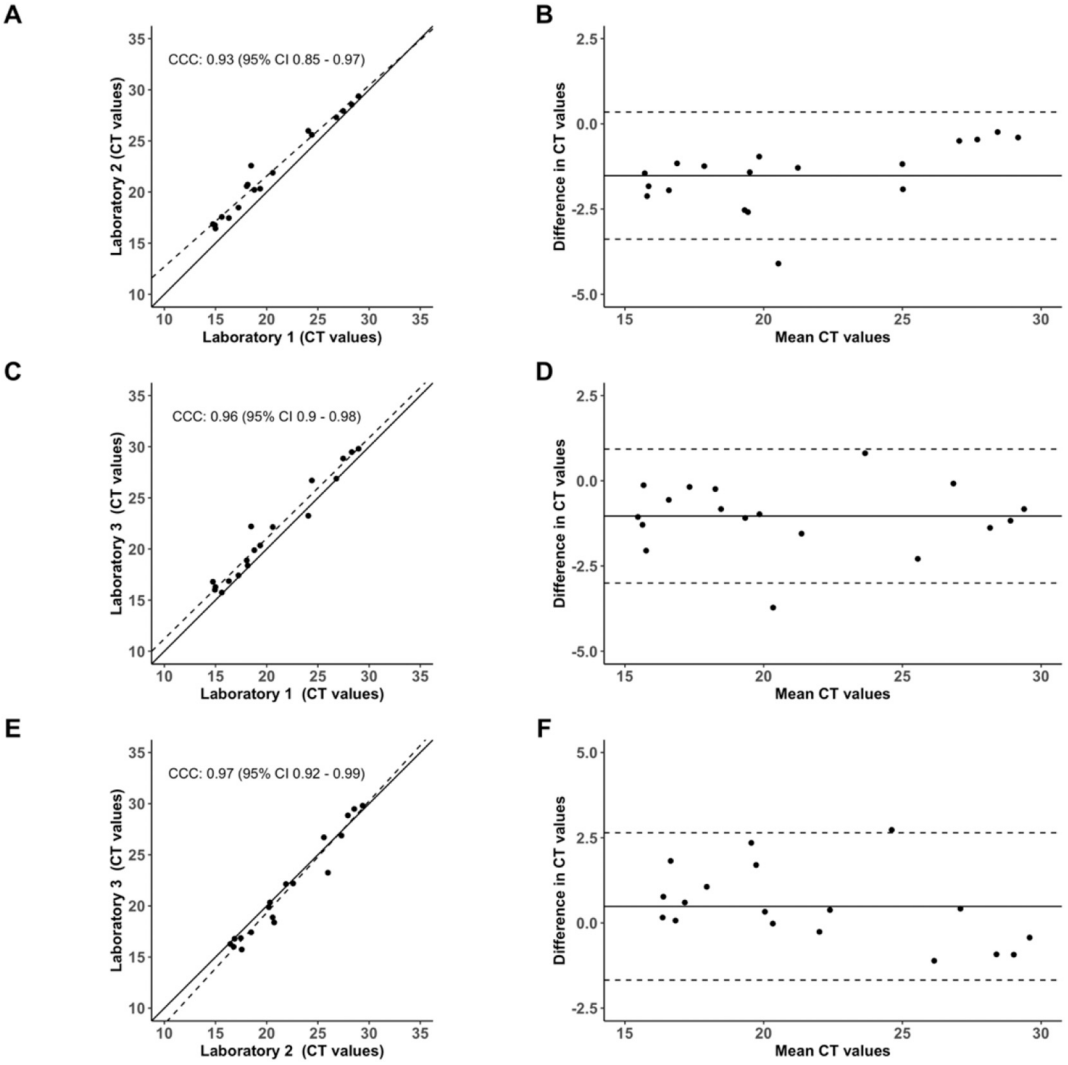

Fig. 3. Reproducibility of the POMV segment 8 RT-qPCR assay assessed by the three collaborating laboratories using an inter-laboratory comparability panel (n = 30). Concordance or agreement plots (panels on the left) show the results (C_T values) obtained by the different collaborating laboratories. A best-fit regression *line* (dashed) is compared with the perfect concordance *line* (solid). Bland-Altman plots (panels on the right) show the difference in C_T values on the y axis (solid line: mean difference, dashed lines: ± 1 standard deviation) and the mean C_T values obtained on the samples by each collaborating laboratory. Panels show reproducibility results for laboratories 1 and 2 (A, B), 1 and 3 (C, D) and 2 and 3 (E, F).

complex - PB2, PB1 and PA encoded by segments 1, 2 and 5 - showing lower transcription levels than segments 3, 7 and 8. A transcription study of ISAV-infected fish found similar results with particularly high expression values for segments 3 and 8 (Valenzuela-Miranda et al., 2015). The authors of this study even suggested that segment 3 should be evaluated as a more sensitive target detecting ISAV. Expression of segment 3 was also comparatively high in POMV-infected cells (Samsing et al., 2020a). However, it was decided to target segments 7 and 8 because of their comparatively high transcription levels *in vivo* (Samsing et al., 2020a).

Samples from different tissues and different populations of infected fish should be used to further validate a new assay's diagnostic performance. In this study, the diagnostic characteristics of the POMV segments 7 and 8 assays were initially assessed using samples of head-kidney from a population of experimentally infected fish. The reproducibility of the selected assay - POMV segment 8 - was also evaluated with a panel of gill homogenate samples from a different POMV experimental infection. Regarding different tissues, viral gene expression patterns appear to be similar between head kidney and liver in POMV-infected fish (Samsing et al., 2020a), with segment 8 presenting the highest level in both. In addition, the comparison of the POMV segment 5, 7 and 8 assays using the samples from the inter-laboratory comparability panel derived from gill tissue, showed consistently lower C_T values for segment 8, suggesting a higher abundance of this transcript in the gills. Considering different fish populations, the present study used samples from experimentally infected fish to assess diagnostic performance. A different experimental population was used to assess reproducibility. However, both cohorts originated from experimental infections and as such, our newly developed assay should be further assessed using field samples. Ideally, these would include tissues from sub-clinical, clinical and vaccinated salmonids, together with samples obtained from other wild fish species acting as reservoirs of POMV, such as wild pilchards.

Diagnostic test characteristics of the newly developed assays were estimated using latent class analysis because none of the available reference methods were assumed to be diagnostically perfect, so-called gold standard (Caraguel et al., 2019). In other words, the true infectious status of these fish was unknown and this is why we chose to use LCM to estimate DSe and DSp. If the true status of these samples was known, simple cross-tabulation tables could be used. In this study, however, the sample size was relatively small, which means that the estimates from the LCM were heavily reliant on the prior information or beta

distribution given to each parameter in the model. This is why model test parameters were elicited from experts information, which is a common method to set priors for DSe and DSp in latent class analysis (Ekong et al., 2017).

The present study focused primarily on developing a more sensitive RT-qPCR that could be used to detect POMV in subclinical or apparently healthy fish. A highly sensitive and highly specific PCR method had been previously developed to detect POMV targeting segment 5 of the viral genome. However, genomic studies examining the host-pathogen interactions during the infection with POMV in Atlantic salmon identified segments of the viral genome highly expressed during the early stages of infection and were used here as diagnostic targets. Targeting POMV segment 8 consistently increased the analytical sensitivity of the assay, while maintaining analytical specificity and delivering reproducible results. The assays targeting segments 7 and 8 also showed an increase in diagnostic sensitivity compared to the segment 5 assay. These assays were able to detect apparently healthy or subclinical fish which were negative to the current PCR method targeting segment 5. These results show that RNA sequencing technologies can help understand the host response to infection, but also guide and inform the development of new molecular diagnostic tools. Further investigation and validation of the new assays using field samples of farmed Atlantic salmon is recommended prior to the implementation of the assay for surveillance purposes.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2021.737404.

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