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Establishment of a piscine myocarditis virus (PMCV) challenge model and testing of a plant-produced subunit vaccine candidate against cardiomyopathy syndrome (CMS) in Atlantic salmon *Salmo salar*

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ABSTRACT

Cardiomyopathy syndrome (CMS) is a severe cardiac disease occurring in the grow-out sea phase of farmed Atlantic salmon with approximately 100 outbreaks annually in Norway. Piscine myocarditis virus (PMCV) is believed to be the causative agent of CMS. There is no vaccine available to control CMS, partially because PMCV withstands propagation in known cell cultures. In the present study, we selected the putative capsid protein of PMCV as the candidate antigen for immunization experiments and produced it in the plant *Nicotiana benthamiana* by transient expression. The recombinant PMCV antigen formed virus-like particles (VLPs). To evaluate the efficacy of the plant made VLP vaccine, a PMCV infection model was established. In an experimental salmon vaccination trial, the VLP vaccine triggered innate immunity, and indicative but not significant inhibition of viral replication in heart, spleen and kidney tissues was observed. Similarly, a reduction of inflammatory lesions in cardiomyocytes and subendocardial infiltration by mononuclear leukocytes were observed. Therefore, there was no difference in efficacy or immune response observed post the plant made PMCV VLP antigen vaccination.

Taken together, this study has demonstrated that plant made VLP antigens should be investigated further as a possible platform for the development of PMCV antigens for a CMS vaccine.

1. Introduction

Aquaculture is an important source for protein-based food supply. This has intensified fish farming, which has led to the fast spread of infectious diseases in aquaculture worldwide. Diseases of aquaculture organisms lead to reduced health and welfare for these organisms, economic losses and environmental consequences such as disease transmission to the wild fauna, as well as wide use of antibiotics and chemicals in efforts to control the diseases (Barrett et al., 2020).

The piscine myocarditis virus (PMCV) is a small, non-enveloped icosahedral virus with a single, linear double-stranded RNA genome with three open reading frames (ORF1–3), where the first two code for a putative 91 kDa capsid protein and an RNA-dependent RNA polymerase, respectively. PMCV, tentatively assigned to the *Totiviridae* family, is assumed to be the causal agent of cardiomyopathy syndrome (CMS) observed in both farmed and wild Atlantic salmon (*Salmo salar*) (Haugland et al., 2011; Løvoll et al., 2010; Poppe and Seierstad, 2003). CMS was first observed in 1985 (Amin and Trasti, 1988), but is today present in all areas along the Norwegian coast where salmon farming takes place. CMS occurs in the grow-out sea phase of farmed salmon, typically late in the production cycle, with a median time of 16 months from sea transfer to diagnosis (Bang et al., 2013). The histopathological lesions of the heart appear first in the atrium, later also in the ventricle, and are seen as infiltration of mononuclear cells in subendocardium in

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the spongiform parts, accompanied by degeneration and necrosis of spongious myocardium (Bruno et al., 2013). Diseased fish have signs of severe circulatory disturbances that develop slowly before clinical signs are observed; however, the cardiac changes may cause rupture of the wall of the atrium or sinus venosus and consequently sudden death (Bruno and Poppe, 1996; Ferguson et al., 1990).

The presence and load of RNA from PMCV is highly correlated to the prevalence and severity of CMS lesions (Haugland et al., 2011; Løvoll et al., 2010). One study claimed that PMCV replicates in the GF-1 cell line with release of infectious virus to the culture medium, albeit at low levels (Haugland et al., 2011). However, cultivation has not been reproduced in subsequent studies (Garseth et al., 2018). Therefore, Koch's postulates, which require isolation of the virus, have not been fully demonstrated for PMCV as the etiological agent of CMS. Viruses of the *Totiviridae* family are associated with latent infections of fungi and protozoans, where the virus particles remain intracellular and are transmitted during either cell division, sporogenesis or cell fusion (the International Committee on Taxonomy of Viruses (ICTV) Virus Taxonomy (Adams et al., 2014)). The third ORF in the PMCV genome encodes a putative toxin (Haugland et al., 2011), which possibly is an analogue to the killer toxins whose genes are transported in satellite particles for some totiviruses (Dunn et al., 2013). Sequence identity used for species demarcation shows that totiviruses in general are vastly different genetically, possibly reflecting that the host organisms have had long term separation (Dunn et al., 2013). Totiviral sequences have also been detected in organisms such as arthropods and fish (Koyama et al., 2015; Mor and Phelps, 2016).

Currently, there are no reports of protective vaccination against CMS, and although transcriptomic profiling has been performed (Timmerhaus et al., 2011), there are no reports of a specific immune response in salmon to PMCV after experimental infection. To manage the CMS, a vaccine against PMCV could be beneficial. Given that PMCV does not propagate to a sufficient amount in known cell lines, the production of antigens for a subunit vaccine remains as an achievable strategy for immunization.

Molecular farming is the exploitation of whole plants or plant cells/tissues cultured in vitro to produce vaccine antigens and biopharmaceuticals against human and animal diseases as well as valuable recombinant proteins (Clarke et al., 2017; Daniell et al., 2015; van Eerde et al., 2019). This has been established as an economically viable alternative to mainstream production systems such as microbes and mammalian cells cultivated in large-scale bioreactors. The use of plants for the development and production of recombinant vaccines offers several advantages. a) Green plants use free solar energy and capture CO_2 making them an environmentally friendly production platform. b) Plant-based systems are more economical as plants can be grown cheaper on a larger scale than in other systems, e.g., mammalian cells where media costs increase in line with the production scale and eliminate any economic benefits of large-scale manufacturing (Chan et al., 2016). c) They lack the undesirable components found in conventional systems, e.g., endotoxins in bacteria or hyperglycosylated proteins produced by yeast, and reduce the risk of contamination with harmful pathogens, which is present when working with vertebrate cell cultures. d) Plant-based systems are extremely versatile and possess the ability to carry out complex post-translational modifications (Daniell et al., 2015; Marsian and Lomonossoff, 2016).

To date, many vaccine antigens, monoclonal antibodies (mAbs), such as the ZMapp™ antibody against Ebola virus (Qiu et al., 2014), and other biopharmaceuticals have been produced in plants (Fischer and Buyel, 2020). Significant progress has been made to overcome the bottlenecks, i.e., in the yields of upstream production and downstream processing to gain a sufficient quantity of purified proteins and regulatory issues of plant-made recombinant proteins (particularly vaccines in this case), as well as using good manufacturing practice (GMP). Preclinical and clinical studies of a large number of plant-made human and animal recombinant pharmaceutical proteins are encouraging and have demonstrated the feasibility, efficacy, and safety of plant factories for vaccines, monoclonal antibodies, and therapeutic protein production (Chan et al., 2016; Clarke et al., 2017; Fischer and Buyel, 2020).

Experimental challenge models represent an essential tool to study disease development in fish. The experimental models are designed to mimic authentic infections in a controlled environment and are typically performed by either intraperitoneal (i.p.) injection of the pathogen, or cohabitation of naïve fish together with shedder fish, or by immersion. The i.p. route of infection does, however, to a lesser degree mimic the natural route of infection compared to challenge by cohabitation and immersion. The models should therefore be validated with respect to representing real-life conditions for the infectious pathogen.

We report in the current study the production in *N. benthamiana* of a VLP-forming PMCV antigen and its performance in a fish immunization and subsequent challenge trial. To our knowledge, this is the first report of PMCV antigen produced in plants and tested in immunization trials, and that may contribute to the development of a CMS vaccine. Green plants could have potential as an antigen production platform for fish vaccines.

2. Material and methods

2.1. Plasmid construction

The PMCV ORF1 coding sequence was combined with a His_6-tag at the C-terminus, codon optimized for *N. benthamiana* expression and synthesized (GeneArt, Regensburg, Germany). The sequence was inserted into the plant transient expression vector pEAQ-*HT*-DEST1 (Sainsbury et al., 2009) using Gateway cloning, essentially as described previously (Clarke et al., 2017). *AttB1* and *attB2* recombination sites flanking the PMCV ORF1 were generated by PCR using primers 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGAACCTAACACCT CTGT-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTAGTGGT GGTGATGGT GAT-3′ generating the pEAQ-*HT*-DEST1-PMCV transient expression vector (Fig. 1A).

2.2. Production of PMCV antigen in Nicotiana benthamiana

The vector pEAQ-*HT*-DEST1-PMCV was introduced into *ElectroMAX™ Agrobacterium tumefaciens* LBA4404 cells (Invitrogen, USA) by electroporation, as described previously (Clarke et al., 2017). Agroinfiltration was performed (Sainsbury et al., 2009) using an inhouse vacuum-based agroinfiltration setup (Clarke et al., 2017) on six-week-old *N. benthamiana* plants, grown in a confined S2 safety level. For initial expression analysis, agroinfiltrated leaves were harvested at different days post agroinfiltration (dpi). Total protein extraction, SDS-PAGE and immunoblot analysis were performed as described in van Eerde et al. (2020). The PMCV antigen was detected with monoclonal anti-polyhistidine antibodies (His-1; Sigma Aldrich, St Louis, USA), followed by goat anti-mouse HRP-conjugated Abs (Santa Cruz Biotechnology, Dallas, USA).

2.3. Extraction and purification of recombinant PMCV capsid protein

Frozen leaves, harvested six to seven days after infiltration, were ground to powder with a liquid nitrogen-cooled mortar and pestle. The powder was mixed with extraction buffer (1 mM EDTA, 20 mM β-mercapto-ethanol, 0.5% (*v*/v) Triton X-100, 50 mM NaCl, 100 mM Tris pH 9) with a ratio of 4 mL buffer for every gram of leaf powder. The mixture was incubated at room temperature for 15 min and filtered through two layers of Miracloth (Merck, Darmstadt, Germany). The filtrate was centrifuged for 20 min at 4 °C at 15000*g*, after which the supernatant was applied to a DEAE Sepharose Fast Flow (GE Healthcare, Uppsala, Sweden) column pre-equilibrated in a solution containing 50 mM Tris pH 9 and 50 mM NaCl. The flow-through containing the PMCV capsid protein was carefully mixed with an equal volume of a solution

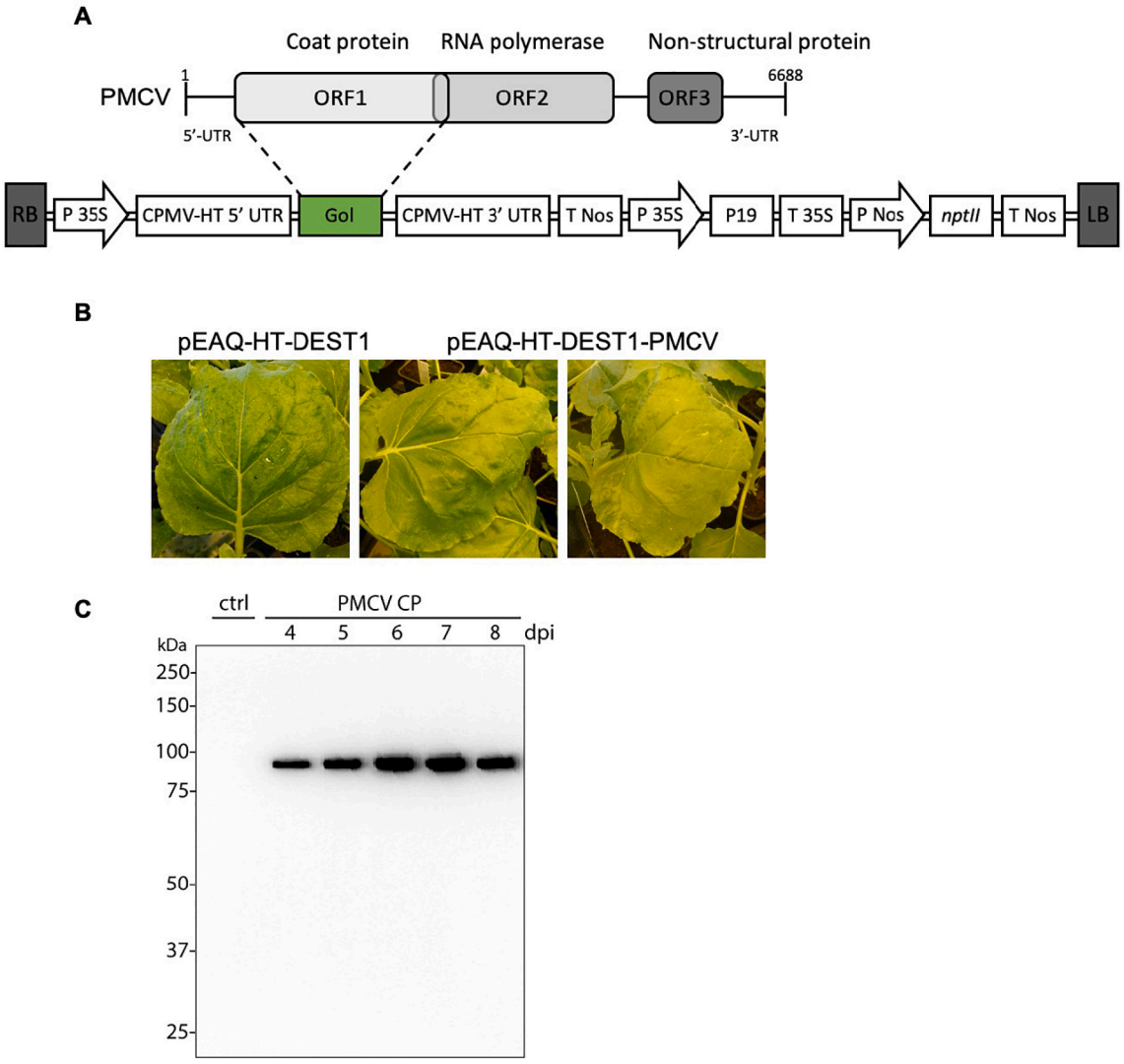

Fig. 1. Transient expression of PMCV capsid protein in *N. benthamiana*. (A) The sequence coding for the capsid protein in the PMCV genome was introduced with an additional C-terminal His_6-tag into the pEAQ-*HT*-DEST1 vector (Sainsbury et al., 2009). RB/LB, right and left borders; 5'UTR and 3'UTR, 5′ and 3′ untranslated regions derived from Cowpea mosaic virus (CPMV); 35S P, 35S promoter; 35S T, 35S terminator; Nos P, nopaline synthase promoter; Nos T, nopaline synthase terminator; P19, suppressor of gene silencing; *nptII*, neomycin phosphotransferase II gene conferring kanamycin resistance; GoI, gene of interest. (B) Phenotype of *N. benthamiana* leaves at 7 dpi respectively agroinfiltrated with pEAQ-*HT*-DEST1 empty vector and pEAQ-*HT*-DEST1-PMCV. (C) Immunoblot analysis with samples of total protein extracts from infiltrated *N. benthamiana* leaves harvested 4, 5, 6, 7 or 8 days post-infiltration (dpi), and a negative control. Each sample contains a total protein amount of 0.5 μg.

containing 16% (*w/v*) polyethylene glycol (PEG) 8000 and 0.6 M NaCl. The mixture was incubated at 4 °C overnight and centrifuged at 10000*g* for 30 min at 4 °C. The supernatant was discarded, after which the pellet containing the PMCV capsid protein was thoroughly suspended in Tris-buffered saline. The suspension was centrifuged at 10000*g* for 10 min to remove insoluble components. For further concentration the supernatant was again subjected to PEG 8000 precipitation as above, after which the pellet was redissolved in a smaller volume of phosphate-buffered saline also containing 10% (*v/v*) glycerol.

For the preparation of a control vaccine, an extract was prepared from non-infiltrated *N. benthamiana* leaves following a similar procedure as described above, but the sample was not applied to the DEAE sepharose column before the PEG 8000 precipitation steps, resulting in a solution of a mixture of *N. benthamiana* proteins with a total protein concentration adjusted to equal that of the PMCV capsid protein solution.

2.4. Transmission electron microscopy (TEM) analysis

Antigen samples were deposited on formvar-coated copper grids and negatively stained with 2% uranyl acetate before TEM analysis, using the JEM-1400 microscope (Jeol).

2.5. PMCV infection model construction

The study included five challenge experiments at VESO Vikan aquatic research facility (Vikan, Norway) (Table 1). The initial two pilots aimed to generate piscine orthoreovirus (PRV) free infectious material for downstream use. The fish, Atlantic salmon smolts, 50–200 g at challenge, were unvaccinated, seronegative for antibodies against bacteria, and confirmed free of infectious pancreatic necrosis virus (IPNV), infectious salmon anemia virus (ISAV), salmon pancreas disease virus (SPDV), PRV and PMCV before initiation of the study. The fish were acclimatized prior to challenge, kept under 12:12 or 24:0 L:D light regime in 12 °C sea, brackish or fresh water. The fish were anesthetized

Table 1
Overview of study design in infection model establishment. Challenge route, fish size, water quality, duration and stress.

	I. P.	I. M.	Cohabitant, % shedders	Fish size, g	Salinity, ppm	Duration, weeks	Stress, low O_2
Pilot 1	●		0	50–200	34	6–15	
Pilot 2	●		0	50–200	34	8	
Study 1	●	●	0	50–200	34	10	
Study 2	●		50	50–200	34	24	
Study 3	●		0	50–200	34	8	●

by bath immersion in benzocaine chloride (2 ± 5 min, 0.5 g / 10 L water) prior to handling, and euthanized using concentrated benzocaine chloride (1 g / 5 L water). The fish were observed at least once per day. No fish died during the trial. Experiments had been approved by the Norwegian Food Safety Authority according to the European Union Directive 2010/63/EU for animal experiments.

Pilot 1: The original tissue homogenate injected into the experimental fish originated from a field CMS outbreak found to be PMCV positive and PRV negative by qPCR. The fish were sampled in the period 6–15 wpc. Heart, spleen and head kidney were tested for presence of PMCV and PRV by qPCR and histology.

Pilot 2: In the second pilot, material from one individual fish from a field CMS outbreak was used, PMCV positive and PRV negative by qPCR. Prior to injection the tissue was applied to polyclonal anti-PRV sigma 1 antibody (Finstad et al., 2012). Briefly, the polyclonal anti-PRV sigma 1 antibody was diluted in 1% skimmed milk in TBS (1:3000) and tissue was applied to the diluted antibody in a humidity chamber at 4 °C overnight. Then gentamicin was added. Sampling was conducted at 2, 4, 5, 6, 7 and 8 wpc. Heart, spleen and head kidney were sampled in RNAlater and tested for presence of PMCV and PRV by qPCR.

Study 1: The effect of i.p. versus i.m. injection of PMCV containing tissue for transmission to cohabitant fish was tested. The experiment lasted 10 weeks. All fish tested negative for PRV.

Study 2: The long-term cohabitant experiment lasted for 24 weeks. In accordance with the prior challenge experiment the cohabitant fish started to become PMCV positive at 9 wpc (Table 2).

Study 3: To try to increase the shedding and thus possible transmission, the experimentally injected fish were artificially stressed by three weekly 30 min pulses of low oxygen tension. Samples were collected in weeks 6 and 8 (Table 3).

2.6. Industrial scale fish trial and pathological analysis

An overview of the experiment design is shown in Table 4. Batches of purified recombinant PMCV capsid/VLP (0.8 mg/mL) were prepared as a water-in-oil formulation with adjuvant (antigen solution: adjuvant = 1:3); the commercial adjuvant used was Montanide™ ISA 761 VG. Control vaccine batches made up with irrelevant proteins from a *N. benthamiana* extract were produced similarly to the PMCV capsid/VLP and prepared similarly as a water-in-oil formulation. The industrial scale fish trial was divided into two parts. Ninety fish were divided into three groups (PBS, control vaccine and PMCV vaccine) and each group of 30 fish was respectively immunized with PBS, control vaccine and PMCV vaccine by 0.1 mL (20 μg antigen/fish) i.p. injection. Six weeks after immunization, all fish were challenged by i.m. injection with 0.1 mL of original tissue homogenate that was positive for PMCV by qPCR and originated from a field CMS outbreak; however, since PMCV resists propagation in cell culture the amount of infectious virus was not known. Heart, spleen, and kidney tissues were obtained at 0, 4 and 8 wpc for qPCR measurement of PMCV replication and innate antiviral response. For unchallenged groups, 25 fish were immunized with PMCV vaccine and 25 fish were left without any treatment. At 0 and 4 wpi, heart, spleen and kidney tissues were randomly obtained and prepared for immune related gene detection by RT-qPCR.

Ventricle and atrium of heart tissue from random fish were collected at 0 and 8 wpc. Pathological sections were prepared and stained with haematoxylin and eosin for histological evaluation. The histologic lesions were scored according to the standard shown in Table 5.

Table 2
Positive cohabitant fish in long-term cohabitant experiment.

Cohabitants	Cont.	9 wpc	10 wpc	12 wpc	24 wpc
PMCV pos/total	0/3	1/3	1/3	1/3	13/24
PRV pos/total	0/3	0/3	0/3	0/3	0/24

2.7. Total RNA extraction and quantification of mRNA expression in fish tissues

Total RNA was isolated from tissue samples that were homogenized in QIAzol Lysis Reagent (Qiagen, Hilden, Germany) using 5 mm steel beads and TissueLyser II (Qiagen) for 2 × 5 min at 25 Hz, added chloroform and centrifuged. The aqueous phase was collected and used for automated RNA isolation using RNeasy Mini QIAcube Kit (Qiagen) as described by the manufacturer. RNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The Qiagen OneStep kit (Qiagen) was used for RT-qPCRs. The input was standardized to 100 ng (5 μL of 20 ng/μL) of total RNA per reaction. Prior to RT-qPCR, the template RNA was denatured at 95 °C for 5 min. The RT-qPCR targeted PRV segment S1 and was performed using previously described conditions (Wessel et al., 2015). The samples were run in duplicate. The primers used for PMCV were as follows: PMCV-F, 5′-AGGGAAVAGGAGGAAGCAGAA-3′; PMCV-R, 5′-CGTAATCCGACATCATTTT GTGA-3′. Other primers used for qRT-PCR analysis are listed in Table 6. Four weeks after challenge, heart, spleen, and kidney tissues were collected and extracted for total RNA. PMCV RNA expression was detected using PMCV primers with SYBR. EF1α and EF1αB genes were used as housekeeping genes (Olsvik et al., 2005). The same method was used to measure antiviral gene expression.

2.8. Statistics

The data were analyzed using an unpaired, two-tailed Student's *t*-test. *P* values below 0.05 were regarded as being significant for all analyses (*, $p < 0.05$; **, $p < 0.01$).

3. Results

3.1. Production of PMCV capsid protein in N. benthamiana plants

The putative capsid protein encoded by PMCV *orf1* (Uniprot e3w911, (Haugland et al., 2011)) was selected as the antigen in a possible subunit vaccine against CMS. Similar capsid proteins have been shown to be able to assemble into virus-like particles which have potential as a subunit vaccine (Yin et al., 2010). To produce the PMCV capsid protein in *N. benthamiana*, a plasmid vector for transient expression of PMCV *orf1* was designed and constructed (Fig. 1A). The PMCV antigen was then produced in *N. benthamiana* leaves by vacuum-based agroinfiltration (Fig. 1B). Plant materials were collected at different days after vacuum infiltration (dpi) to evaluate the optimal expression level for the PMCV capsid protein in *N. benthamiana*. Using immunoblotting with anti-polyhistidine antibodies on total protein extracts, a single band of an

Table 3
PMCV transmission in fish stressed by low oxygen exposure.

	Stress treatment	Injected fish (i.p.)	Cohabitant fish	PMCV Ct 6 wpc (i.p.)	PMCV Ct 8 wpc (i.p.)	Histoscore 8 wpc
Tank A	No	50	0	21.14	25.13	0.25
Tank B	Yes	13	50	20.73	22.37	0.5–0.75

Table 4
Industrial scale fish trial design.

Tank	Group	Immunization (i.p.)	Sampling at challenge	Challenge (i.m.)	Sampling at 4 wpc/wpi	Sampling at 8 wpc/wpi
Tank 1	PMCV vaccine	30	6	24	6	6
	Control vaccine	30	6	24	6	6
	PBS	30	6	24	6	6
Tank 2	PMCV vaccine	25	–	–	6	6
	Untreated	25	–	–	6	6

Table 5
Standard pathological statistic classification.

Tissue	Score	Description
Heart	0	No pathological findings, or slightly increased number of leukocytes
	1	One or a few focal lesions, increased number of leukocytes
	2	Several distinct lesions and small to moderate increase in number of leukocytes
	3	Multifocal to confluent lesions and moderate to severe increase in number of leukocytes
	4	Severe confluent lesions comprising >75% of the tissue and massive leukocyte infiltration
Liver	0	No pathological finding, sparse focci circulatory disturbance (congestion sinusoids)
	1	Moderate diffuse circulatory disturbance (congestion sinusoids), sparse degeneration of hepatocytes
	2	Sparse - moderate multifocal necrosis
	3	Moderate-severe multifocal necrosis
	4	Diffuse severe necrosis

apparent molecular weight of roughly 95 kDa could be observed in all samples (Fig. 1C), which corresponds well with the expected molecular weight of 92 kDa for the His_6-tagged PMCV capsid protein. The highest accumulation of PMCV antigen was observed at 6–7 dpi, which was used for the subsequent production of PMCV antigen (Fig. 1C).

3.2. VLP formation of recombinant PMCV capsid antigen

PMCV antigen produced in *N. benthamiana* plants was extracted in soluble form from leaves and purified by a combination of anion-exchange chromatography and PEG precipitation (Fig. 2A). A total of 1.2 mg of antigen could be isolated from 250 g of infiltrated leaf material. To determine whether the plant-produced PMCV capsid protein was able to assemble into virus-like particles, samples were analyzed by negative-staining TEM. This showed the presence of round particles with a diameter ranging between 40 and 50 nm (Fig. 2B).

3.3. Construction of PMCV infection model and industrial scale fish trial

To construct a PMCV infection model, two in vivo pilots and three in vivo studies were performed according to the experimental design (Table 1).

Pilot 1: Experimental fish were injected with tissue homogenate originating from a field CMS outbreak, collected from diseased fish and found to be PMCV positive and Piscine orthoreovirus (PRV) negative by qPCR. Heart, spleen and head kidney were sampled at 6–15 weeks post challenge (wpc) and tested for presence of PMCV and PRV by qPCR. At 6 wpc, two of three fish were positive for PMCV in spleen and head kidney, while only one fish was positive in the heart samples (data not shown). However, the injected fish became qPCR positive for PRV, while the control fish stayed negative, indicating that that the original field material did contain PRV, although being negative for PRV by qPCR (data not shown).

Pilot 2: Experimental fish were injected with material from one individual fish from a field CMS outbreak, which was PMCV positive and PRV negative by qPCR. The injection material was treated with anti-PRV sigma 1 antibody and gentamicin prior to injection. Heart, spleen and head kidney were sampled at 2, 4, 5, 6, 7 and 8 wpc and tested for presence of PMCV and PRV by qPCR. The samples were negative for PRV by qPCR, and from 2 wpc started to be positive for PMCV (data not shown). Material sampled at 7 wpc was used for the subsequent challenge experiments.

Challenge study 1: The effect of i.p. versus intramuscular (i.m.) injection of PMCV containing tissue for transmission to cohabitant fish was tested. At 9 wpc, the first cohabitant fish became PMCV positive; however, at 10 wpc only 3/6 and 2/6 of the cohabitants of i.p. and i.m. injected fish were PMCV positive, respectively (data not shown). No difference in the ability to transmit the infection was observed for i.m. or i.p. injected fish. A long-term cohabitant experiment with i.p. injected fish was therefore pursued to study the ability to transmit PMCV to cohabitants over time.

Challenge study 2: A long-term cohabitant experiment lasting for 24 weeks was performed. At 24 wpc, only 13/24 (54%) of the cohabitant fish were positive for PMCV (Table 2). Histopathological examination of hearts from cohabitant fish showed lesions in line with those of CMS, but the histological scores were modest. There was a correlation between the Ct and histopathological scores, where the fish with the lowest viral load (Ct 34.6) had the lowest histoscore (0.25) and the individual with highest viral load (Ct 20.5) had the highest histoscore (1.0) (data not

Table 6
Primers used for qRT-PCR in this study.

Gene name	GenBank ID	Size (bp)	Forward (5′ - 3′)	Reverse (5′ - 3′)
EF1a	BT072490.1	77	CACCACCGGCCATCTGATCTACAA	TCAGCAGCCTCCTTCTCGAACTTC
EF1αB	BG933897	57	TGCCCCTCCAGGATGTCTAC	CACGGCCCACAGGTACTG
Stat1a	GQ325309.1	127	CGGTGGAGCCCTACACTAAG	GGGATCCTGGGGTAGAGGTA
Rig-I	FN178459.2	142	GACGGTCAGCAGGGTGTACT	CCCGTGTCCTAACGAACAGT
Mx	NM001139918	173	CGGTGATAGGGGACCAGAGT	CTCCTCACGGTCTTGGTAGC
Viperin	NM001140939	101	AGCAATGGCAGCATGATCAG	TGGTTGGTGTCCTCGTCAAAG

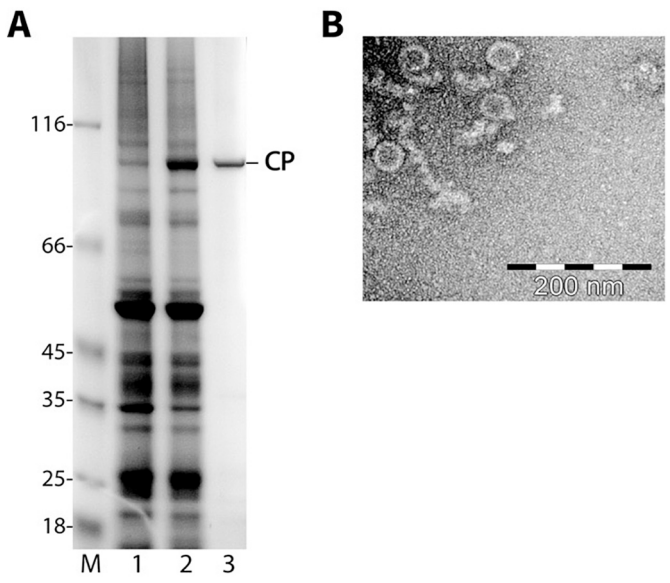

Fig. 2. Purification and characterization of recombinant PMCV capsid protein. (A) SDS-PAGE analysis of extracts from infiltrated leaves from (1) negative control and (2) pEAQ-*HT*-DEST1-PMCV, and (3) purified PMCV capsid protein and (M) Unstained Protein Molecular Weight Marker (Thermo). (B) Electron microscopy analysis of recombinant PMCV capsid protein. VLPs purified from *N. benthamiana* leaves were negatively stained with uranyl acetate and visualized by TEM. Scale bar is 200 nm.

shown).

Challenge study 3: To try to increase the shedding and thus possible transmission, the fish were stressed by exposure to low oxygen for 30 min, 3 times in weeks 5 and 7. Samples were collected in weeks 6 and 8. No cohabitant fish became PMCV positive after the stress treatment, i.e., in line with studies 1 and 2, where no cohabitants had become positive at this time post injection of shedder fish. The histoscores, performed for samples collected at 8 weeks, were higher for the group that had been stress-treated than for the control group (Table 3).

Following challenge, the pilots and studies, a PMCV infection model was established and used for experimental challenge of fish at reproduceable and large scale according to the design in Table 4. Pathological changes were recorded and graded in naive fish, PBS vaccinated fish, control vaccinated fish and PMCV vaccinated fish at 6 weeks before PMCV challenge and at 0, 4 and 8 wpc. Ventricle and atrium of fish heart were collected for histological analysis at 0 and 8 wpc. Heart, spleen and kidney tissues were obtained at 0, 4 and 8 wpc or weeks post injection (wpi) in different groups with or without PMCV infection for RT-qPCR detection to evaluate the load of viral RNA and innate antiviral immune response.

3.4. Histological changes in challenged fish

Histological changes in immunized and PMCV challenged fish were evaluated in epicardium, spongiosum, compactum and atrium compartments of the heart as well as in liver tissue. The standardized scoring is showed in Table 5. According to the scatter plots shown in Fig. 3, liver tissue was not significantly affected by the PMCV infection (Fig. 3A). The overall histopathological score for heart showed that fish immunized with plant produced PMCV capsids had a lower score than the PBS and control vaccine groups at 8 wpc, but the difference was not significant (Fig. 3B). Specifically, spongiosum and atrium parts of the heart had more serious lesions and the PMCV vaccine group showed lower scores for these compartments than the other groups at 8 wpc (Fig. 3E, F).

To visually observe the pathological damage to fish heart by PMCV infection, histological analysis was performed. Ventricle and atrium of heart tissue were randomly obtained from infected fish at 0 and 8 wpc (Fig. 3G). The collected tissues were sectioned and stained with haematoxylin and eosin. Minor inflammatory lesions in cardiomyocytes and subendocardial infiltration by mononuclear leukocytes were observed in the tissues with PMCV vaccine immunization at 8 wpc.

3.5. PMCV replication in immunized fish post challenge

In heart tissue, the viral RNA levels of the PMCV vaccine group were reduced compared to the levels in the control vaccine group at 8 wpc (Fig. 4A). The difference was not statistically significant. In spleen, viral RNA transcripts in fish in the PMCV vaccine group were slightly lower than in the control group at 4 wpc (Fig. 4B), and similar in kidney 8 wpc (Fig. 4C). The differences were not statistically significant.

3.6. Innate antiviral immune responses

To study if the PMCV vaccine triggers the host innate immune system, expression analysis of antiviral immune system related receptors was performed for PMCV vaccine and to untreated groups after 4 weeks after immunization (Fig. 5). The PMCV vaccine increased both RIG-I and STAT1 mRNA expression levels in heart and spleen tissues at 4 wpi compared to the untreated fish (Fig. 5A, B). In kidney, the RIG-I and STAT1 mRNA expression levels in kidney were reduced in the PMCV vaccine antigen group compared to the untreated group at 4 wpi (Fig. 5A, B). However, none of these differences were significant because of the within-group differences.

Expression analyses of the antiviral effectors Mx (Fig. 6A) and Viperin (Fig. 6B) were carried out in heart and kidney at 4 wpc and 8 wpc for the PBS, control vaccine and PMCV vaccine groups. The Mx and Viperin mRNA levels in the PMCV vaccine group were higher than those in the PBS or control vaccine group at 4 wpc. At 8 wpc, no difference was observed. There were large within-group differences. There was no effect seen from the vaccination.

4. Discussion

In the present study PMCV capsid protein produced in *N. benthamiana* plants was used for immunization of Atlantic salmon against CMS. First, an experimental PMCV challenge model was established. After an initial trial where the tissue used for challenge, collected from a field outbreak of CMS, was found to contain PRV after challenge of fish. In the further challenges the fish were found free of PRV and thus histopathological changes were associated with PMCV. The histopathological lesions seen in injected fish were considered as typical for CMS; however, the score was moderate to low, 1–1.5 of a maximum score of 3. No mortality was induced. The histopathology score was lower than what has been reported for earlier transmission studies where 36% of the injected fish had score 3 at the peak of the infection (Timmerhaus et al., 2012). This could indicate that the PMCV strain in our experiments could be of low virulence; however, variation in PMCV virulence is not reported from the field and genetically the virus is assessed as rather homogenous (Wiik-Nielsen et al., 2013).

We found PMCV in a low prevalence, approximately 30%, in cohabitant fish after 9–10 weeks with cohabitation. Even after 24 weeks, only 54% of the cohabitant fish were positive for PMCV by qPCR. This could indicate that the shedding of PMCV is slow and low, or the virus is not easily transmitted through water or by direct contact, or the susceptibility of the experimental fish was low. The experimental conditions we used, where possible other concomitant virus infections or vector hosts for PMCV were absent or kept at a minimum level, lessened the severity and transmissibility of the infection compared to field observations. In order to increase shedding, the experimentally injected fish were artificially stressed by three weekly 30 min pulses of low oxygen tension; however, this did not increase the number of fish infected by cohabitation, but slightly increased the histoscore in the injected fish.

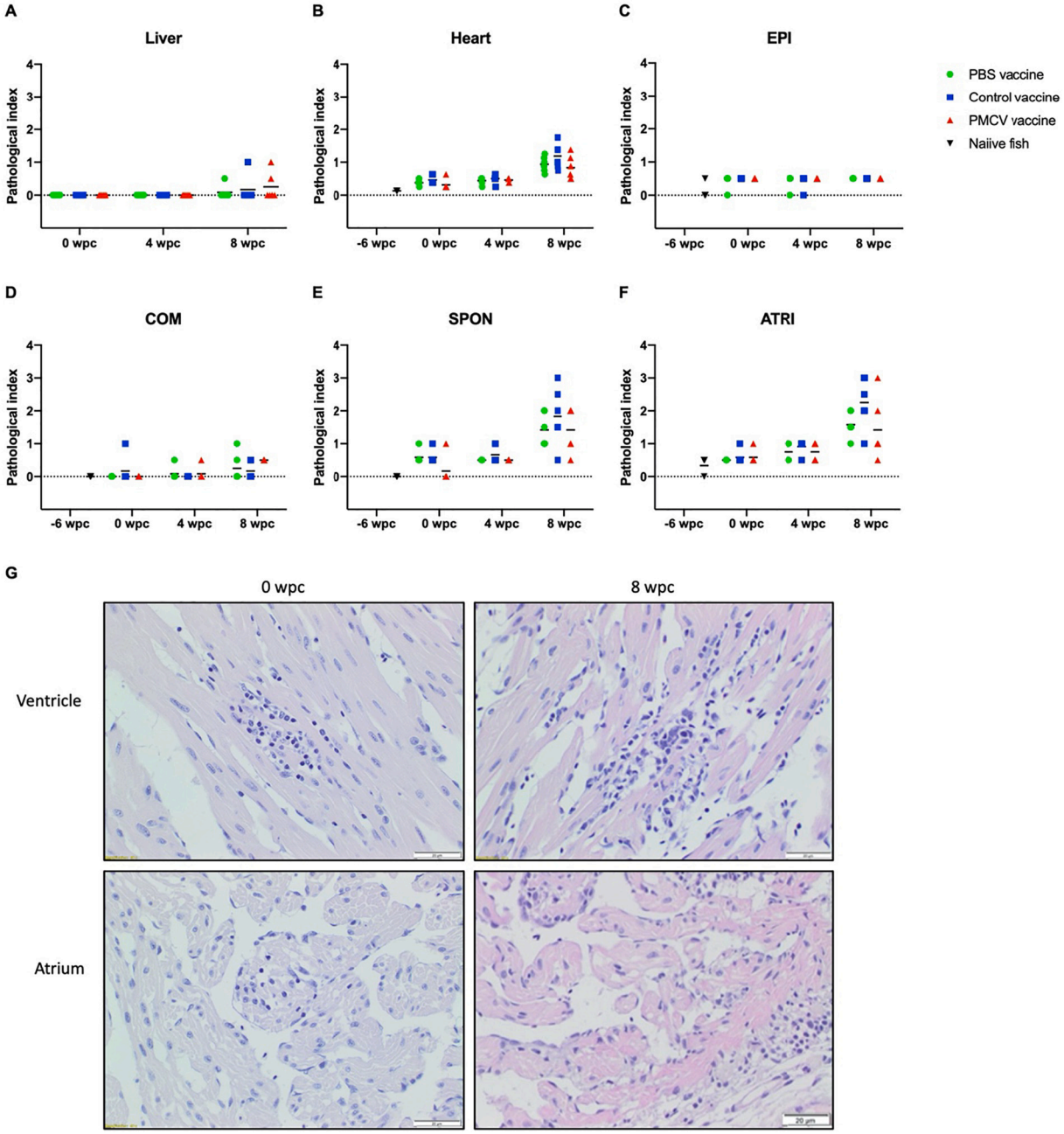

Fig. 3. Tissue lesion analysis. (A-F) The pathological changes of different structures of heart and liver were recorded and classified. The classification standard is shown in Table 5. (G) Histological analysis of ventricle and atrium of heart tissue post PMCV vaccine immunization and subsequent PMCV challenge. Histological observed lesions in cardiac atrium and spongy ventricle at 0 wpc and 8 wpc. Minor inflammatory lesions in cardiomyocytes and subendocardial infiltration by mononuclear leukocytes. The sections were stained with haematoxylin and eosin. Scale bar (applies to all panels) = 20 μm.

Thus, we did not find indication of increased transmissibility due to stress.

PMCV was horizontally transmitted in the present experimental conditions, but to a very low extent. In former studies, the conclusion was that the primary route for transmission of CMS in the field is horizontal (Bang et al., 2013). We could not verify this with the experimental challenge model we used. This could indicate that in the field, the virus spreads through water using mechanisms absent or low in experimental settings. Epidemiological studies have found increased probability of occurrence of CMS with time post sea transfer, infection pressure, cohort size, and previous diagnosis of heart and skeletal muscle inflammation (HSMI) (Bang et al., 2013). None of these factors were reflected in the experimental conditions; the time after the transition from fresh to sea water was short, the cohort size of experimental fish was a tiny fraction of that of a commercial net, and PRV, i.e., the etiological cause of HSMI, was not present. However, the present experimental model makes it possible to study factors of CMS development related only to PMCV.

PMCV has been detected in mucus, feces and salmon lice from diseased fish (Hellebø et al., 2014). Dual infection of PRV and PMCV is common (Wiik-Nielsen et al., 2016). In a study of viral co-infections in farmed Atlantic salmon where samples were collected 5–22 months post sea-transfer, PRV was found to be ubiquitous, but there was no negative correlation between the presence of PMCV and PRV (Wiik-Nielsen et al., 2016), which could seem paradoxical. PRV is a strong inducer of interferon and thus causes interference, i.e., restriction of replication of

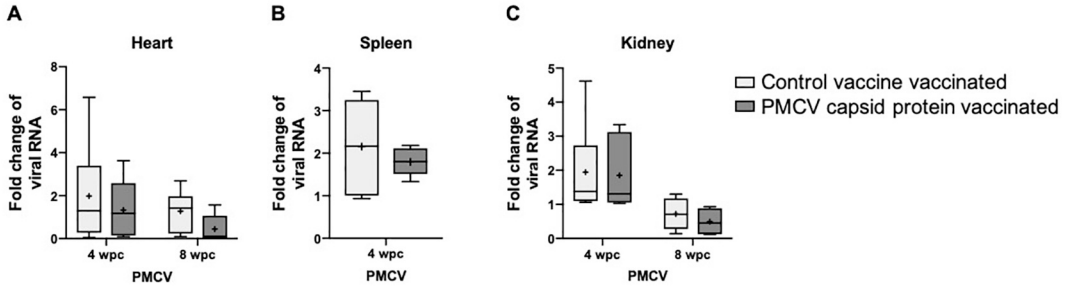

Fig. 4. PMCV replication in different tissues in fish after immunization and challenge. Fold change expression of PMCV replication in heart (A), spleen (B) and kidney (C) at 4 wpc. Data of qRT-PCR shown as mean ± SD of 6 fish. Significance was calculated in relation to the PBS control group. EF1α and EF1αB genes were used as housekeeping genes (Olsvik et al., 2005) and the relative transcription levels are represented as fold induction relative to the transcription level of PBS control group. + represents mean.

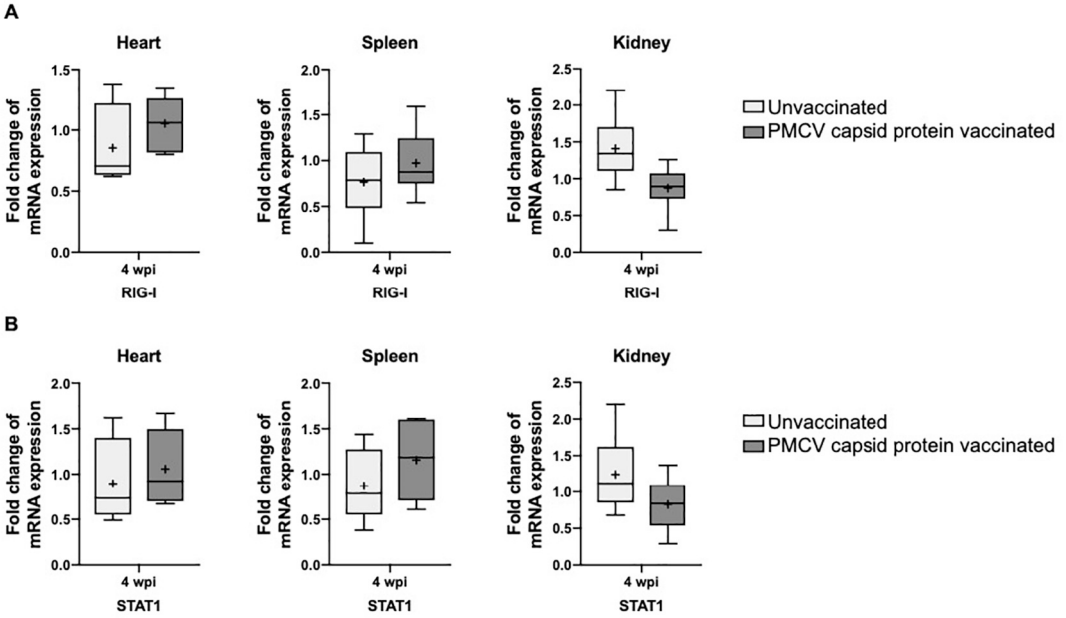

Fig. 5. RIG-1 and STAT-1 expression in different tissues after immunization and challenge. Fold change expression of RIG-I (A), and STAT1 (B) in heart, spleen and kidney tissue at 4 wpc. Data of qRT-PCR shown as mean ± SD of 6 fish. Significance was calculated in relation to the PBS control group. EF1α and EF1αB genes were used as housekeeping genes and the relative transcription levels are represented as fold induction relative to the transcription level of PBS control group. + represents mean.

other interferon sensitive viruses such as ISAV and IPNV (Lund et al., 2016; Vendramin et al., 2018). This could be explained by PMCV appearing only after the IFN induced by PRV has returned to basic levels, or that PMCV has an efficient anti-IFN counter response. The first point is unlikely due to the wide distribution of these viruses. Given the general properties of *Totiviridae*, i.e., viruses associated with infections of fungi and protozoans, an alternative explanation would be that PMCV does not primarily infect salmon cells, but another organism, yet undescribed, associated with farmed salmon. This could explain the lack of interference to PRV, hitherto lack of reported PMCV induced specific acquired immune responses, and lack of successful cultivation of the virus in fish cells.

The results from prior experiments where only the input material, and not the experimental fish after injection, were tested for purity of PMCV should be carefully interpreted. In the early transmission experiments performed for CMS (Bruno and Noguera, 2009; Fritsvold et al., 2009) there were no controls of the viral flora of the inoculum, due to lack of knowledge of other viruses such as PRV and Gill pox virus. Furthermore, in the present experiment we used material that was found to be PRV negative according to the qPCR results in the first pilot experiment, but nevertheless the experimental fish became PRV positive.

The main effects of CMS and HSMI are necrosis and inflammatory cells affecting different regions of the heart; for CMS the viral load is highly correlated with the development of lesions (Haugland et al., 2011). Furthermore, laser dissection studies indicated that PMCV is almost exclusively present in myocardial lesions (Wiik-Nielsen et al., 2012). Others have, however, found that PMCV is present in all organs tested prior to the heart lesions (Timmerhaus et al., 2012). High correlation between viral load and cardiac histopathology score and sites of lesions suggest that viral cytopathic effect is an important factor for the myocardial lesions (Timmerhaus et al., 2012). On the other hand, lipid content and composition in the feed may have an immunomodulatory effect, resulting in a milder and delayed immune response after PMCV infection, and significant reduction in tissue damage during CMS outbreaks (Martinez-Rubio et al., 2014). This indicates that the immune response is an important factor for the development of lesions.

To produce low cost PMCV vaccine and to test the established experimental model for PMCV, the putative PMCV capsid protein was produced in *N. benthamiana* and purified to obtain VLPs for a PMCV vaccine. To our knowledge this is the first time PMCV antigens have been produced in a plant expression system. Plant derived PMCV

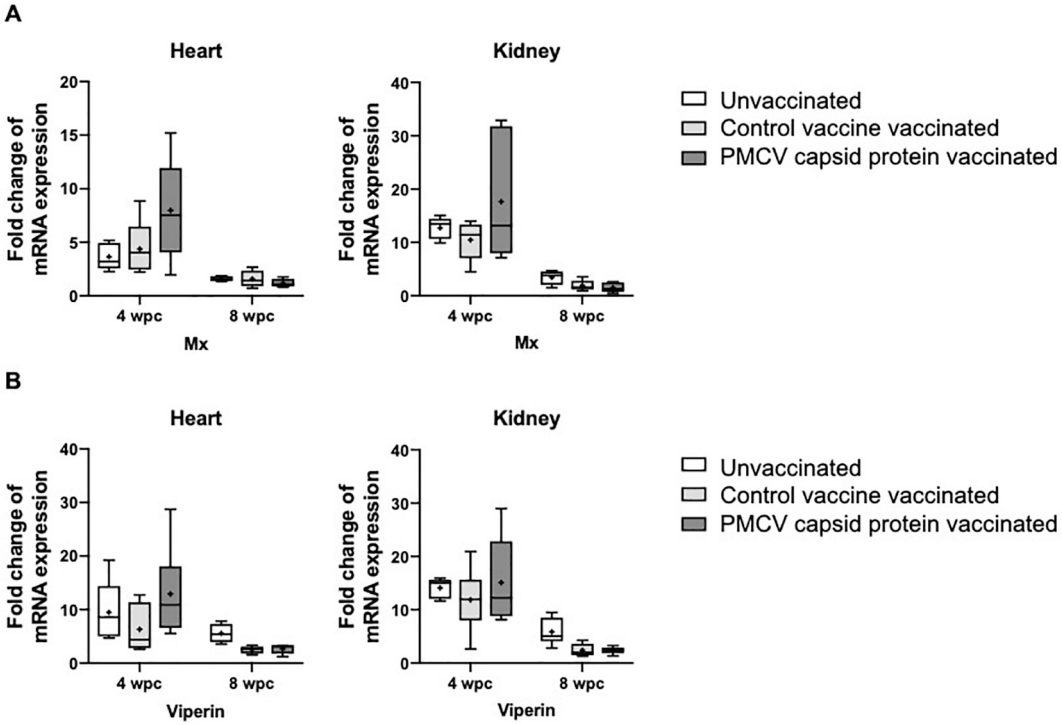

Fig. 6. The Mx and Viperin response to PMCV vaccine immunization. Fold change expression of Mx (A) and Viperin (B) in heart, spleen and kidney tissue at 4 and 8 wpc. Data of qRT-PCR are shown as mean ± SD of 6 fish. The relative transcription levels were normalized to the average transcription level of EF1α and EF1αB genes and are represented as fold induction relative to the transcription level at 0 wpi of each group. + represents mean.

vaccine is environmentally friendly, low-cost, low-toxicity and safe. Therefore, it is of high interest to test if a plant-produced subunit PMCV vaccine has sufficient antigenicity to induce protection to CMS.

The present study successfully produced highly purified PMCV antigen with high efficiency in *N. benthamiana*. This provides the potential capacity to produce low-cost CMS vaccine. The PMCV capsid protein produced in tobacco corresponds well to the size and shape of the full PMCV virions (Haugland et al., 2011). The TEM analysis suggests that the recombinant PMCV capsid protein retains the ability to self-assemble in virus-like particles, a property that may be relevant for the immunogenic properties of the protein.

We found that the plant derived PMCV vaccine reduced the pathological lesions in different structures of heart tissue, reduced the load of viral RNA and increased the expression of the RIG-I and STAT1 receptors. However, none of these changes were statistically significant. RIG-I is a primary pattern recognition receptor in cytoplasm sensing viral RNA in an innate immune system. RIG-I regulates IFN to induce antiviral effectors reducing viral replication. STAT1 is a core molecule in the JAK-STAT signaling pathway regulated by IFN to transmit an antiviral signal to downstream adaptors linking to antiviral effectors. The PMCV replication was not significantly inhibited in vivo after immunization with plant made PMCV capsid antigens.

5. Conclusions

In this study, a PMCV challenge model has been established for industrial scale fish experiments. The model was used to test a plant made PMCV subunit capsid antigen vaccine. Although the vaccinated fish had less histological changes and lower viral RNA loads than the controls, the differences between the groups were not significant.

Author statement

The authors declare that there is no conflict of interest for the current study.

Author contributions

ER, AvE, IØ and JLC conceived and designed the study. HS, ER and JLC wrote the manuscript with contributions, review and editing by all co-authors. HSS, SH and IH participated in the experimental plan for vector construction and conducted all the plant agroinfiltration work. AvE performed the protein analysis and purification. ER designed the vaccination and challenge trials for the PMCV vaccine antigen while MI and ML performed the vaccination and challenge trials for PMCV at VESO Vikan, Namsos, and collected all samples. HS did all the qRT-PCR analysis for samples collected from the PMCV fish vaccination trial and received from IØ. IØ prepared sample collection and SCW carried out the histochemical assay.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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