Cardiomyopathy Syndrome of Atlantic Salmon (*Salmo salar* L.) Is Caused by a Double-Stranded RNA Virus of the *Totiviridae* Family[▽]

Øyvind Haugland,¹ Aase B. Mikalsen,^{1¶} Pål Nilsen,^{2¶} Karine Lindmo,^{2¶} Beate J. Thu,¹
Trygve M. Eliassen,² Norbert Roos,³ Marit Rode,² and Øystein Evensen^{1*}

Norwegian School of Veterinary Science, Department of Basic Sciences and Aquatic Medicine, P.O. Box 8146 Dep., N-0033 Oslo, Norway¹; Pharmaq AS, P.O. Box 267, N-0213 Oslo, Norway²; and Department of Molecular Biosciences, University of Oslo, P.O. Box 1041, Blindern, Oslo, Norway³

Received 12 October 2010/Accepted 4 March 2011

Cardiomyopathy syndrome (CMS) of farmed and wild Atlantic salmon (*Salmo salar* L.) is a disease of yet unknown etiology characterized by a necrotizing myocarditis involving the atrium and the spongy part of the heart ventricle. Here, we report the identification of a double-stranded RNA virus likely belonging to the family *Totiviridae* as the causative agent of the disease. The proposed name of the virus is piscine myocarditis virus (PMCV). On the basis of the RNA-dependent RNA polymerase (RdRp) sequence, PMCV grouped with *Giardia lamblia* virus and infectious myonecrosis virus of penaeid shrimp. The genome size of PMCV is 6,688 bp, with three open reading frames (ORFs). ORF1 likely encodes the major capsid protein, while ORF2 encodes the RdRp, possibly expressed as a fusion protein with the ORF1 product. ORF3 seems to be translated as a separate protein not described for any previous members of the family *Totiviridae*. Following experimental challenge with cell culture-grown virus, histopathological changes are observed in heart tissue by 6 weeks postchallenge (p.c.), with peak severity by 9 weeks p.c. Viral genome levels detected by real-time reverse transcription (RT)-PCR peak earlier at 6 to 7 weeks p.c. The virus genome is detected by *in situ* hybridization in degenerate cardiomyocytes from clinical cases of CMS. Virus genome levels in the hearts from clinical field cases correlate well with the severity of histopathological changes in heart tissue. The identification of the causative agent for CMS is important for improved disease surveillance and disease control and will serve as a basis for vaccine development against the disease.

Cardiomyopathy syndrome (CMS) is a disease primarily affecting adult Atlantic salmon 14 to 18 months after sea transfer and close to harvest and was first described as a disease entity in Norway in 1985 (2). By 1990, approximately 100 Norwegian fish farms were found to be affected by the disease out of a total of 600 fish farms in Norway (2, 11). Later, CMS was diagnosed in the Faroe Islands and Scotland (31, 33), and cases with resemblance to CMS have also been reported from Canada (3). The number of farms where fish have been diagnosed with CMS in Norway has been 75 to 85 per year over the last decade (4). CMS has also been reported in wild, migrating Atlantic salmon and is considered a potential threat to wild salmon populations (31, 33).

Fish suffering from CMS may suddenly die without clinical signs of disease, while symptoms can be as nonspecific as abnormal swimming behavior. At autopsy, findings include cardiac tamponade with blood in the pericardial sac and moderate to pronounced ascites (2, 11). CMS is diagnosed by histopathology with severe inflammation and degeneration of the spongy part of the myocardium (ventricle) and with similar changes in the atrium (11). Myocyte degeneration and inflammatory changes are not frequently seen in the compact layer of the heart and always occur later than changes of the spongy

parts (12). Circulatory disturbance with multifocal liver necrosis may also occur.

Environmental factors have been hypothesized as the cause of CMS, while recently the transmissible nature of the disease was demonstrated (12). Changes typical of CMS occurred in postsmolts of salmon 6 weeks after injection of organ homogenate (heart and kidney) from fish with CMS (12), indicative of an infectious etiology.

Here, we show that the causative agent of CMS in Atlantic salmon is a virus with a double-stranded RNA (dsRNA) genome of 6,688 bp. Following experimental challenge, viral genomes are detected by real-time reverse transcription (RT)-PCR at 2 weeks postchallenge (p.c.), peaking 6 weeks postchallenge. The peak viral load precedes peak histopathological changes in heart tissue by approximately 2 weeks. The virus genome is also detected by *in situ* hybridization in necrotic cardiomyocytes. Phylogenetic analysis based on the RNA-dependent RNA polymerase (RdRp) clustered the virus with infectious myonecrosis virus of shrimp and *Giardia lamblia* virus (GLV), a member of the family *Totiviridae*. We propose that the virus be named piscine myocarditis virus (PMCV). The identification of a causative agent for CMS is an important step toward better disease control and development of a vaccine against CMS.

MATERIALS AND METHODS

Virus propagation in cell culture. Heart tissue from freshly dead Atlantic salmon (*Salmo salar* L.) was collected from a clinical outbreak of CMS (the diagnosis was confirmed by histopathological examination). The heart tissue was homogenized, followed by centrifugation at 4,000 × g and 4°C for 20 min to

* Corresponding author. Mailing address: Norwegian School of Veterinary Science, Department of Basic Sciences and Aquatic Medicine, P.O. Box 8146 Dep., N-0033 Oslo, Norway. Phone: 472 2964500. Fax: 472 2597310. E-mail: oystein.evensen@nvh.no.

¶ A.B.M., P.N., and K.L. made equal contributions to this study.

[▽] Published ahead of print on 16 March 2011.

remove cellular debris, before being processed through a 0.22- μ m filter. The homogenate was subsequently inoculated onto GF-1 cell cultures (5). The GF-1 cell line, derived from the fin tissue of orange-spotted grouper, *Epinephelus coioides*, was grown in plug seal cap culture vessels at 15°C after inoculation in Leibowitz 15 supplemented with 1% L-glutamine (2 mM final concentration), 0.1% gentamicin sulfate (50 μ g/ml final concentration) (all from Sigma Aldrich), and 10% fetal bovine serum (Invitrogen). Development of cytopathic effect (CPE) was monitored at 6, 14, and 21 days postinoculation (p.i.). At 21 days p.i., the supernatant was harvested and passaged further in cell culture. Infected GF-1 cell cultures were also harvested at 7, 14, and 21 days postinoculation and prepared for real-time RT-PCR studies to quantify the relative amounts of virus genome in the cells and in the supernatant over the course of the infection.

A second study was carried out in which heart homogenate (as described above) was inoculated onto CHH-1 cell cultures originating from chum salmon (*Oncorhynchus keta*) heart tissue (20), incubated for 4 h at 15°C, and then washed two times with sterile phosphate-buffered saline (PBS). Fresh medium, Eagle's minimum essential medium (EMEM) supplemented with 1% L-glutamine (2 mM final concentration), 1% nonessential amino acids, 0.1% gentamicin sulfate (50 μ g/ml final concentration) (all from Sigma Aldrich), and 10% fetal bovine serum (Invitrogen), was then added. The cells were grown in six-well cell culture dishes at 15°C. Cell culture supernatants and cell layers (after being washed 2 times in PBS) from triplicate wells were harvested separately at 0, 1, 3, 6, 10, and 14 days p.i. (0 days p.i. is immediately after inoculation removal and PBS wash; negative controls were also harvested at this time). The harvests were prepared for real-time RT-PCR studies to quantify the relative amounts of cell-associated virus and virus in the supernatant over the course of the infection.

Negative staining. Hearts collected from Atlantic salmon suffering from CMS were homogenized with L-15 medium (3 times the weight of the tissue sample) and then centrifuged at 4,000 $\times g$ for 10 min at 4°C. The supernatant was filtered at 0.22 μ m. Fifteen milliliters of the homogenate was transferred to a concentration device (Macrosep 100 kd; Pall) and centrifuged at 2,800 $\times g$ at 10°C for 120 min. The retentate was then transferred to a new concentration device and spun for another 90 min. Five milliliters of the resulting retentate was incubated with 50 μ l Igepal CA-630 (Nonidet P40 substitute; Sigma-Aldrich) and incubated at room temperature for 20 min. The detergent-treated PMCV material was mixed with PBS and 4.5 g of CsCl in a final volume of 12 ml. The gradient was stored at 4°C. One tube (Thinwall; Polyallomer; 13.2 ml; 14 by 89 mm; Beckman) was filled with the gradient solution, placed in an SW41ti rotor (Beckman), and spun at 257,000 $\times g$ at 4°C for 24 h to form a gradient. The gradient was fractionated by bottom puncture in approximately 0.5-ml aliquots, and the refractive index was measured with a pocket refractometer, PAL-RI (Atago). Fraction 3, with a density of 1.3842 g/ml, was subjected to electron microscopy examination. Copper grids (100 mesh; hexagonal) coated with a carbon-stabilized Formvar film were placed on 5- μ l drops (fraction 3) for 5 min. The samples were then chemically fixed for 1 min by floating the grids on drops of 2.5% glutaraldehyde solution (0.1 M cacodylate buffer, pH 7.4) and subsequently washed by floating them on 5 drops of distilled H₂O for a total of 8 min. Staining was performed by placing the grids on drops of 3% uranyl acetate (in H₂O) for 3 min, after which the excess staining solution was removed from the grid (Whatman filter paper grade 1). The grids were dried at room temperature before being imaged. Pictures were taken at 80 kV using a Philips CM200 transmission electron microscope equipped with a Quemesa 11-megapixel on-axis charge-coupled device (CCD) camera (Olympus Soft Imaging Solutions). The fractions examined were also assayed for the presence of virus by real-time RT-PCR methods as described below.

Cloning and identification of the virus genome. A sequence-independent approach to viral-genome sequencing and further identification and characterization of the virus were performed as described by Djikeng et al. with minor adjustments (7, 8). The method includes a random-priming (RP) strategy in combination with sequence-independent single-primer amplification (SISPA), coupled with construction of a plasmid library, sequencing of random library clones, and assembly into a nearly full-length viral genome. Supernatant from GF-1 cultures inoculated with a homogenate from a clinical outbreak of CMS were carefully collected after 14 days of culture, centrifuged to remove cellular debris, and processed through a 0.22- μ m filter. The viral particles were concentrated by ultracentrifugation (Beckman Coulter Optima L-80 XP Ultracentrifuge). Approximately 30 ml supernatant was centrifuged in 10-ml aliquots at 100,000 $\times g$ for 4 h in a SW41 rotor (Beckman Coulter). Nearly all of the cell culture medium was carefully removed before pellets containing virus were pooled and resuspended in a total volume of 200 μ l of a combination of remaining old and fresh cell culture medium. To enrich for viral particles and eliminate residual nucleic acid contaminants in the culture medium, 100 units of DNase I (Qiagen) and 10 μ g/ml RNase A (Sigma-Aldrich; catalog no. R6513) in RDD

buffer (Qiagen) was added to the viral resuspension and incubated at 37°C for 1 h. RNA was isolated immediately after nuclease treatment using the QIAamp viral kit (Qiagen) according to the manufacturer's instructions, but without using carrier RNA.

Single-stranded cDNA was synthesized using a SuperScriptIII reverse transcriptase kit (Invitrogen) and a random chimeric primer (1 μ M) containing a fixed sequence of 20 nucleotides (nt) and a random hexamer attached to its 3' end (FR26RV-N primer: 5'-GCCGGAGCTCTGCAGATATCNNNNN-3'). RNA, deoxynucleoside triphosphates (dNTPs), and the FR26RV-N primer were mixed and incubated at 95°C for 2.5 min prior to cDNA synthesis to avoid problems caused by a possible secondary structure of the RNA. The reaction mixture was then immediately transferred to ice before the enzyme mixture was added, and reverse transcription was performed as described in the random-primer protocol. Following heat inactivation of the enzyme mixture, RNA complementary to the synthesized cDNA was removed by adding 2 units of *Escherichia coli* RNase H (Invitrogen) and incubation at 37°C for 20 min. Double-stranded cDNA was synthesized by Klenow reaction using a combination of the 2nd-strand buffer components of the Universal RiboClone cDNA Synthesis System (Promega) and exonuclease-free Klenow polymerase (Invitrogen; catalog no. AM2006). The reaction mixture contained 10 μ l first-strand reaction mixture, 20 μ l second-strand buffer, 2.5 μ l acetylated bovine serum albumin (BSA), 10 U Exo-Klenow polymerase, 0.2 μ M random primers (FR26RV-N), and finally 15 μ l nuclease-free water. The reaction mixture was incubated at 37°C for 30 min before inactivation of the enzyme by incubation at 70°C for 10 min. Finally, a single-primer PCR using 5 μ l of the double-stranded cDNA, standard *Taq* polymerase (Invitrogen), and the FR20RV primer (5'-GCCGGAGCTCTGCAGATATC-3') was performed. The smear of PCR products was separated by 1% agarose gel electrophoresis. Products in the 500- to 1,000-bp size range were excised from the gel and purified using a QIAquick Gel Extraction Kit (Qiagen). The PCR products were ligated into the pCR 2.1 vector using a Topo TA cloning kit (Invitrogen) and transformed into competent OneShot TOP10 bacterial cells (Invitrogen). Approximately 300 clones were picked and sequenced using a commercial service (GATC Biotech).

Assembly of the viral genome. Sequence reads were trimmed to remove vector sequence and amplicon primer sequence, as well as low-quality sequence, and assembled into contigs using the Contig Express program of Vector NTI advance 11.0 (Invitrogen). Despite the filtration- and nuclease treatment-reduced presence of nucleic acids from GF-1 cells, contaminating RNA and DNA species inevitably remain and thus are amplified. The sequences, both contigs and singletons, were therefore first analyzed for repetitive sequences using Tandem Repeats Finder (<http://tandem.bu.edu/trf/trf.html>). The remaining sequences without repeats were subjected to BLAST analysis (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). A putative sequence identity was determined based on BLAST similarity, and sequences matching a cellular origin were discarded. PCR primers were designed from the remaining potential virus-specific sequences to screen cDNA from GF-1 cultures inoculated with virus originating from fish with CMS, piscine reovirus (PRV), and salmonid alphavirus 3 (SAV3). Primers resulting in a specific product based on cDNA from CMS-related cultures were further tested on heart tissue from diagnosed CMS field outbreaks and CMS-challenged Atlantic salmon (described below). In summary, two long contigs of 4.0 kb and 2.4 kb were shown to resemble the genome of PMCV. Based on results from BLASTX analysis, it was likely that the two contigs might be merged into one long contig. Two primers, PMCV-bridge F (5'-AGCAGGAGCAACAGCACAC-3') and PMCV-bridge R (5'-TACCCGCCAAATGGTACTTC-3'), were designed to span the region between the two contigs. A PCR product was obtained and sequenced as described above, and the sequence revealed 96 new bases separating the two smaller fragments. The total contig comprised 6,535 bp after this step. Based on the sequence information, primers were designed, and the sequence was resequenced using a commercial service (GATC Biotech).

Determination of 5'- and 3'-terminal sequence by SMART-RACE. To obtain the full-length sequence of the viral genome, rapid amplification of cDNA ends (RACE) was performed using the SMARTer RACE cDNA Amplification Kit (Clontech). RNA was isolated from heart tissue of Atlantic salmon and from the supernatants of GF-1 cultures inoculated with PMCV. First-strand cDNA synthesis was performed following the protocol with random priming according to the instructions of the manufacturer. After first-strand synthesis, the product was diluted with 20 μ l Tricine-EDTA buffer. RACE PCRs were then performed using the Advantage 2 Polymerase Mix (Clontech), the Universal primer A mix (UPM) provided in the SMARTer RACE kit, and genome-specific primers at the 5' and 3' ends of the sequence, followed by nested PCR using the UPM and nested PMCV primers. The following virus-specific primers were used: 5'RACE (5'-ATGGCAGCCTGCTGCTGCTCCGTTGC-3'), 5'RACE-nested (5'-CAACGCCGGCCGGGACTACCGAAAT-3'), 3'RACE (5'-CCAGAACACGCGG

TABLE 1. Scoring criteria for sections

Score	Types of changes ^a
0	No pathological findings or negligible no. of inflammatory cells present
0.1–0.9	Increasing no. of inflammatory cells per focus and moderate myocyte necrosis per focus
1	One or a few distinct focal lesions with cardiomyocyte necrosis and distinct infiltration of leukocytes (1–5 per focus)
1.1–1.9	Increasing no. of lesion individually increasing in size per focus
2	Several distinct lesions and increased no. of leukocytes
2.1–2.9	Multifocal myonecrosis with increasing no. of inflammatory cells
3	Multifocal to confluent lesions and moderate to severe increase in no. of leukocytes
3.1–3.9	Increasing no. of foci toward diffuse lesions; inflammation increases, as do endothelial hyperplasia
4	Severe confluent lesions comprising >75% of the tissue and massive leukocyte infiltration

^a Scoring criteria have been modified from reference 12 to obtain a continuous scale. The changes observed by histological examination have been indicated on a visual analog scale (0 to 4) based on the criteria in the table.

AACCGAGGAACACG-3'), and 3' RACE-nested (5'-GTCGCTAGCACCTG GGCGAAGATGG-3'). The fragments in both reactions were gel purified, cloned, and sequenced as previously described.

Characterization of the genome. Nucleic acids isolated from the supernatants of GF-1 cell cultures inoculated with heart homogenate from fish with CMS were subjected to enzymatic analysis to determine the character of the viral genome. Additionally, nucleic acids from cell cultures infected with a double-stranded RNA virus (infectious pancreatic necrosis virus [IPNV]) and a single-stranded RNA (ssRNA) virus (infectious salmon anemia virus [ISAV]) were included as controls. The amount of virus genome for the three viruses was adjusted to be within a crossing-point (Cp) value of 20 ± 3 . Nucleic acids from all three viruses were pooled and subjected to nuclease treatment for 1 h. Digestions were performed using either 50 U mung bean nuclease (MBN) (New England BioLabs), 2 μ g of RNase A (Sigma-Aldrich) under both low-salt (10 mM) and high-salt conditions (300 mM), and finally 10 U DNase I (Qiagen). Digestion with MBN was performed at 30°C, that with RNase A at 37°C, and that with DNase I at room temperature. One aliquot of pooled viral genomes was left untreated at 37°C for 1 h. All treatments were performed in duplicate, and following nuclease treatment, all treatments were immediately cleaned up using the RNA cleanup protocol in the RNeasy Mini Kit (Qiagen). Nucleic acids from all treatments were treated equally and finally eluted in 30 μ l RNase-free water, and 8 μ l of each eluate was used for the production of cDNA utilizing the SuperScript III Platinum Two-Step qRT-PCR Kit with SYBR green (Invitrogen). Finally real-time RT-PCR was performed in duplicate on the LightCycler 480 instrument (Roche). Virus-specific primers used for real-time RT-PCR were as follows: IPNV-F (5'-GAAACGCCGACATCGTCA-3'), IPNV-R (5'-AGGG CTCGGGAGAATTTTC-3'), ISAV-F (5'-GCTACACAGCAGGATGCAGA TG-3'), and ISAV-R (5'-ATGCCGGAAGTCGATGAAC-3'). PMCV primers are described below.

Screening of field material. Heart tissue samples from three different fish farms were collected for comparison to examine the association between clinical cases of CMS (with confirmed histopathology) and the presence of virus as assessed by real-time RT-PCR. Sample 1 (S1), Atlantic salmon ($n = 40$) originating from a suspected outbreak of CMS, was collected at the time of slaughter. The two other samples (S2 and S3) included fish ($n = 10$) collected from one suspected case of CMS based on clinical observations and fish ($n = 10$) from one site previously confirmed positive for heart and skeletal muscle inflammation (HSMI) by histopathological examination. Samples from all sites, including the heart ventricle and atrium, were fixed by submersion in 10% phosphate-buffered formalin and allowed a minimum of 48 h for fixation. The specimens were then embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin (H&E) according to standard procedures. The sections were scored using criteria described in Table 1.

Parallel samples were preserved in RNAlater, kept under cold conditions for 2 to 3 days, and then transferred to -20°C . Samples were prepared as described below and examined for the presence of PMCV by real-time RT-PCR as described below.

In situ hybridization. *In situ* hybridization was performed on paraffin-embedded heart tissue from clinically diseased Atlantic salmon, sample S2 (see above). The samples were sectioned and mounted on poly-L-lysine-coated slides, dewaxed in xylene 3 times for 2 min each time, and rehydrated in solutions with decreasing ethanol concentrations (2 times at 100%, 96%, 70%, and 50%; 2 min in each solution). Finally, the slides were rinsed in RNase-free water and washed twice for 5 min each time in PBS (10 mM Na-phosphate buffer, pH 7.4, with 0.9% NaCl added). All solutions until the posthybridization washes were prepared using RNase-free water, and unless otherwise stated, all subsequent PBS washes

were performed twice at room temperature for 5 min each time. Endogenous peroxidase activity was quenched by incubation in 0.1% H_2O_2 in methanol for 20 min at room temperature. The tissues were permeabilized using 40 $\mu\text{g}/\text{ml}$ proteinase K (Sigma-Aldrich) in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4) for 15 min at 37°C. To stop protease digestion, the sections were postfixed with 4% (wt/vol) paraformaldehyde in PBS for 6 min at 4°C, followed by washing them in PBS. Blocking of polar and charged groups was performed by incubating sections at room temperature in triethanolamine (TEA) buffer (0.1 M TEA, pH 8.0) with 0.25% (vol/vol) acetic anhydride added. After incubation for 5 min, additional acetic anhydride was added to reach a final concentration of 0.5% (vol/vol), and the incubation proceeded for another 5 min, followed by washing with PBS. The sections were prehybridized in hybridization buffer (4 $\times$ SSC [600 mM NaCl, 60 mM sodium citrate, pH 7.0], 50% [vol/vol] formamide, 1 $\times$ Denhart's solution [Sigma-Aldrich], and 0.5 $\mu\text{g}/\text{ml}$ salmon sperm DNA [Sigma-Aldrich]) for 1 h at 42°C. Successively, the samples were overlaid with hybridization buffer containing 5% (wt/vol) dextran sulfate (Sigma-Aldrich) and 200 ng/ml digoxigenin (DIG)-labeled probe, placed on a heated surface at 84°C for 10 min, chilled on ice for 2 min, and incubated overnight at 42°C. The probe used was 857 nucleotides in length, homologous to nucleotides 4,658 to 5,514 of the PMV genome, and was labeled with DIG-dUTP by PCR using the PCR DIG Probe Synthesis Kit (Roche). Posthybridization stringency washes were carried out 3 times for 20 min each time at 42°C in 2 $\times$ SSC containing 50% (vol/vol) formamide. The sections were then washed with PBS and blocked with 5% BSA in PBS for 20 min at room temperature. To detect bound DIG-labeled probes, the sections were incubated for 1 h at room temperature with peroxidase-conjugated sheep anti-digoxigenin antibody (Roche; diluted 1:20 in 2.5% BSA in PBS) and stained using a Tyramide Signal Amplification kit (Perkin Elmer) and an AEC staining kit (Sigma-Aldrich) according to the manufacturers' recommendations. The sections were counterstained in Mayer's hematoxylin (Sigma-Aldrich) for 60 s and mounted in aqueous mounting medium (Aquamount; BDH Laboratory).

Challenge experiment. The virus was originally isolated from heart tissue homogenate collected from a clinical outbreak of CMS and filtered through a 0.45- μm filter. Challenge of Atlantic salmon was performed using a cell culture 1st-passage supernatant combined with a cell lysate of the same. This was kept frozen at -80°C until it was used. A total of 80 unvaccinated Atlantic salmon, strain Aqua Gen, were used in the experiment. The fish had been smoltified (seawater adapted) according to standard procedures of the Industrial and Aquatic Laboratory (ILAB), Bergen, Norway, and were kept in 25‰ salt water. The fish were fed according to standard procedures, except for the day of challenge, when the fish were kept off feed. The water temperature was maintained at 12°C with a flow at 0.8 liter/kg fish per minute. The fish were anesthetized and subsequently challenged by intramuscular (i.m.) injection of 0.1 ml inoculum on each side of the fish in the lateral muscle tissue beneath the dorsal fin (total, 0.2 ml per fish). Twenty untreated fish were subsequently marked by clipping the adipose fin and transferred to the same tank as the i.m.-injected fish on the day of challenge. Ten control fish were included to document the absence of any pathological changes at the start of the experiment, and these fish were also used to document freedom from PMCV at the onset of the experiment.

Sampling of tissue from experimentally infected fish and processing procedures. Parallel samples for histology and real-time RT-PCR studies were collected sequentially every second week for the first 6 weeks postchallenge and subsequently every week up to 10 weeks p.c. for the injection-challenged group (6 to 8 fish per sampling). At 6, 8, and 10 weeks p.c., parallel samples were also taken from the cohabitant fish. Heart and muscle tissues were submerged in 10% phosphate-buffered formalin and kept at 4°C for at least 4 days until they were embedded in paraffin according to standard procedures. Sectioning was per-

formed at 4 to 5 μm , and the slides were stained with H&E. For real-time RT-PCR studies, hearts were placed in RNAlater and stored at 4°C for 1 to 3 days before long-term storage at -20°C until RNA isolation. Prior to challenge, 10 fish were sacrificed as negative controls. Histological changes in the CMS-challenged fish were scored using criteria described in Table 1.

Real-time RT-PCR. Total RNA from experiments in GF-1 cells was isolated using TRIzol LS (Invitrogen), while RNA from heart specimens was isolated using the RNeasy minikit (experimental samples) or RNeasy Fibrous Tissue minikit (field samples) (Qiagen) according to the manufacturer's instructions. Homogenization of heart tissues was done in the kit lysis buffer using a mixer mill (Retsch GmbH & Co.). RNA quantification was determined by spectrophotometry using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies), and the optical density at 260 nm (OD_{260})/ OD_{280} ratio was used to evaluate the purity of the nucleic acid samples. Following isolation, total RNA (800 ng) was reverse transcribed into cDNA using the SuperScript III Platinum Two-Step qRT-PCR Kit with SYBR green (Invitrogen) according to the protocol for first-strand cDNA synthesis, except that an initial denaturation of RNA was performed at 95°C for 5 min prior to adding the enzyme mixture. Real-time RT-PCR on samples from experimental challenge and work in cell culture was run on the LightCycler 2.0 instrument (Roche), with all reactions performed in duplicate. The following primers were used: PMCV-F (5'-ACACCAGGTGAC CGAAAAG-3'), PMCV-R (5'-TCCAGTGCCTTGATGTCTG-3'), β -actin F (5'-CCAGTCCTGCTCACTGAGGC-3'), and β -actin R (5'-GGTCTCAACA TGATCTGGGTCA-3'). Real-time RT-PCR on field samples was run on the MXpro 3000P (Stratagene), with all reactions performed in triplicate using the following primers: ORF2-3F (5'-GGAAGCAGAAGTGGTGGAGCGT-3') and ORF2-3R (5'-CCGGTTTTCGCGCCCTTCGTC-3'). The reaction conditions were UDG incubation at 50°C for 2 min and activation of the hot-start polymerase at 95°C for 2 min, followed by 40 to 45 cycles of 95°C for 15 s, primer annealing for 15 s, and extension for 1 min at 60°C. Melting curve analysis was performed to confirm formation of the expected PCR products, and the products from all assays were additionally tested by agarose gel electrophoresis to confirm the correct size of the products. The viral loads in heart specimens following experimental challenge were normalized against endogenous β -actin and expressed as a relative copy number (the fish with the lowest viral load was set to 1). The remaining real-time results were normalized to the input of RNA, and viral loads were presented as Cp values.

The field samples were additionally tested for the presence of both PMCV and PRV with TaqMan MGB probes for detection, using the LightCycler 480 Probes Master on the LightCycler 480 instrument (Roche). The sequences of primers and probes were as follows: PMCV-F2, 5'-AGGGAACAGGAGGAAGCAGA A-3'; PMCV-R2, 5'-CGTAATCCGACATCATTTTGTGA-3'; PMCV-Probe, 5'-TGGTGGAGCGTTCAA-3'; PRV-F, 5'-CCCCATCCCTCACATATGGAT A-3'; PRV-R, 5'-GGTGAATCATCGCCAACTCA-3'; and PRV-Probe, 5'-A TGTCAGGTATTTACC-3'. The reaction mixture contained 10 μl of LightCycler 480 Probes Master, 1 μl of primer-probe mixture (final concentrations, 0.50 μM each primer and 0.2 μM probe), 2 μl of cDNA template, and 7 μl water and was incubated for 10 min at 95°C, followed by 45 amplification cycles (10 s at 95°C, 30 s at 60°C, and 1 s at 72°C).

Statistical analysis. All statistical analyses were performed with the help of GraphPad Prism 5.0 (GraphPad Software Inc.). The level of significance for rejection of the null hypothesis (H_0) was set to a P value of <0.05.

Nucleotide sequence accession number. The complete nucleotide sequence of PMCV was deposited in GenBank under accession number HQ339954.

RESULTS

PMCV propagates in cell culture. Since viruses of the family *Totiviridae* in general are transmitted to new cells only during cell division, sporogenesis, and cell fusion, except for *Giardia-virus*, we tested the ability of PMCV to replicate in susceptible cell lines and to what extent virus is released into the supernatant during replication. GF-1 cells inoculated with filtered homogenate of heart tissue from salmon suffering from CMS showed cytoplasmic vacuoles at approximately 6 days p.i. (Fig. 1b). By 14 days, large vacuoles were present throughout the monolayer (Fig. 1c), and the size of the cytoplasmic vacuoles increased gradually up to 21 days p.i. (Fig. 1d) but never developed toward cell lysis. By real-time RT-PCR, there was a moderate but significant increase of virus genomes from 7 to

14 days p.i. in infected cells, leveling off by 21 days p.i. (Fig. 1e). Interestingly, the export of virus to the supernatant was found to be very limited over the observation period, with no significant rise in copy numbers ($\text{Cp} = 22.8 \pm 0.6$ [standard deviation]) over the incubation period (not shown). The supernatant was harvested and passaged at 21 days p.i., and transfer of the supernatant to fresh cell cultures resulted in similar vacuolization that gradually became weaker up to passage 3, after which CPE was no longer observed, indicating that the virus was present in the cell culture supernatant at low passage numbers.

On this basis, we also tested the ability of the virus to replicate in a cell line derived from heart tissue of chum salmon, focusing particularly on release into the extracellular compartment. While CPE similar to that for GF-1 was not observed when CHH-1 cells were inoculated with the same tissue homogenate, virus was found to replicate in CHH-1 cells and also to be released into the supernatant (Fig. 2a and b). The amount of cell-associated virus dropped from 0 h to 1 day p.i. (Fig. 2a), while there was an increase in the supernatant over the same period (Fig. 2b). We interpret these findings as an indication that cell membrane-associated virus (not yet internalized) was part of the virus measured at 0 h (Fig. 2a), while by 1 day p.i., the virus that had not been internalized appeared in the supernatant, thus causing an increase from 0 h to 1 day p.i. (Fig. 2b). For cell-associated virus, there was a significant increase from 3 to 6 days p.i., with no further increase until 14 days p.i. (Fig. 2a). In the supernatant, there was also an increase from 3 to 6 days p.i. (Fig. 2b), concomitant with the increase in the intracellular compartment (Fig. 2a), although the amount of virus was lower in the supernatant. The virus level did not increase any further over the 14-day incubation period.

Negative staining. Virus purified from infected heart tissue was detected in a fraction with a density of 1.3842 g/ml, which corresponds well to that of established members of the *Totiviridae*. This fraction was also positive by real-time RT-PCR. The virus is approximately 50 nm in diameter (Fig. 3a and b), which is slightly above the size seen for other members of the *Totiviridae*. It is not possible to reveal the details of the virion, but there are indications of structural symmetry on the viral surface (Fig. 3b).

PMCV clusters phylogenetically with GLV of the *Totiviridae*. The complete nucleotide sequence of PMCV is one segment of 6,688 bp. The putative RdRp of PMCV shows homology with viruses of the *Totiviridae* known to infect fungi and parasitic protozoa, and also with infectious myonecrosis virus (IMNV) of shrimp (32) (Fig. 4).

Characteristics and organization of the PMCV genome. The base composition of the positive-strand of PMCV was found to consist of 30.2% A, 20.0% U, 31.9% G, and 17.9% C. The GC content of open reading frame 1 (ORF1) is 56.23%, that of ORF2 is 43.56%, and that of ORF3 is 44.99%, indicating a higher GC content in the 5' end of the genome than in the 3' part. By sequence analysis using the Vector NTI advance software, we found two open reading frames (ORF1 and ORF2) potentially translated as a fusion protein (see below) and one additional nonoverlapping open reading frame (ORF3) on the positive-sense strand (Fig. 5). The putative ORF1 is present on the sense strand as reading frame 1 and at nucleotide positions 445 to 3027. The encoded protein is 861 amino acids (aa), with

FIG. 1. (a to d) Phase-contrast micrographs of CPE in GF-1 cells at 0 (a), 6 (b), 14 (c), and 21 (d) days postinoculation with a heart homogenate sampled from Atlantic salmon clinically diagnosed with CMS. The diagnosis was confirmed by histopathology. (e) Virus replication expressed as Cp values at different times after infection of GF-1 cell cultures. There was moderate increase from day 7 to day 14 postinoculation and no further increase up to day 21. The experiment included three parallels at each time point ($n = 3$). P values are indicated (one-sided t test). d, day(s).

a predicted molecular mass of 91.8 kDa and a pI of 5.4. The translation-predicted polypeptide of 861 aa is slightly longer than the average major capsid protein for totiviruses (approximately 750 aa) but substantially shorter than the 1,606-aa-long ORF1 of IMNV from shrimp. Interestingly, the part of ORF1 from IMNV that is predicted to comprise the coat protein has a molecular mass and pI similar to ORF1 of PMCV (99.3 kDa and 5.4, respectively) (32). Homology search with the predicted amino acid sequence of ORF 1 produced no matches with significant similarity in GenBank, similar to what is found for ORF 1 of IMNV. There are two 2A-like motifs (GDVES NPGP and GDVEENPGP) reported in ORF1 of IMNV that

are likely involved in polyprotein cleavage (26, 26), both of which were not present in PMCV ORF1 (or other parts of the genome of PMCV). Further, the dsRNA-binding motif (DSRM) revealed by a conserved domain search (NCBI) in the extreme N-terminal region of ORF1 of IMNV (32) was not detected in the genome of PMCV.

The putative ORF 2 is present in reading frame +3 at nucleotide positions 3114 to 5291. The amino acid sequence of ORF2 showed similarity to the RdRp of the family *Totiviridae*. The encoded protein of 726 amino acids has a predicted molecular mass of 83.1 kDa and a pI of 9.42. PMCV showed its closest match with the gag-pol fusion protein of GLV and

FIG. 2. Virus replication expressed as Cp values at different times after infection of CHH-1 cell cultures in the cell-associated compartment (a) and in the supernatant (b). Cell cultures were infected with filtered, centrifuged homogenate from infected fish (heart) as described in Materials and Methods. The cells were washed after 4 h of incubation, and samples were collected sequentially, as indicated, postinfection. The 0-h time point is the first sampling after incubation and washing. There was a drop in cell-associated virus from 0 h to 1 day p.i. (a) and a similar increase in the supernatant (b). Similarly, there was an increase from 3 to 6 days p.i. for both cell-associated virus (a) and supernatant (b). The experiment included three parallels at each time point ($n = 3$). P values are indicated (two-sided t test).

Giardia canis virus (E values of $4e-15$). The overlap region between ORF1 and ORF2, 86 nucleotides in length, is of particular interest. The predicated ORF1 and ORF2 in initial analysis resembled two nonoverlapping open reading frames; however, ORF2 of PMCV might be translated as a fusion protein with ORF1 due to ribosomal -1 frameshifting (26), which was also predicted for IMNV (37). This frameshifting is often associated with a “slippery site” with the heptameric nucleotide sequence N NNW WWH, where the incoming reading frame is indicated by spacing and both N and W must be stretches of three identical nucleotides (16, 29, 30). This motif, which permits the translational tRNAs to reengage codons in the -1 frame, was found in the genome of PMCV, six bases upstream of the UAG stop codon of ORF1. This suggests that PMCV ORF1 and ORF2 can be translated as a fusion protein, a coding strategy representative of some of the members of the family *Totiviridae*, and GLV in particular (39). This finding was further substantiated by the fact that there was no -1 in-frame stop codon in the sequence stretch between the heptameric “slippery site” of ORF1 and the standard start codon (AUG) of ORF2, thus making such a frameshift possible.

The gap region between ORF2 and ORF3 is 250 nucleotides in length (Fig. 5). Despite the finding of a heptameric slippery site 208 bp upstream of the stop codon of ORF2, ribosomal -1 frameshifting is not likely to occur, as the stretch of nucleotides revealed several stop codons in all three reading frames on the

positive strand. ORF3 therefore seems to be translated as a separate protein. As far as we know, this third ORF has not been described in any previous members of the family *Totiviridae*. The putative ORF3 is present in reading frame 3 at nucleotide positions 5542 to 6447. The encoded protein is 302 amino acids long, with a predicted molecular mass of 33.4 kDa and a pI of 6.99, and shared no sequence homology with known totiviruses or other viruses. Weak homology (E value = 0.011) was, however, obtained with C-X-C motif chemokine 11, and a conserved domain search (NCBI) revealed a chemokine superfamily motif at the N-terminal end. Chemokines are a group of small and structurally related molecules that regulate cell trafficking of various types of leukocytes through interactions with a subset of 7-transmembrane G protein-coupled receptors (25).

PMCV has a double-stranded RNA genome. The next study was undertaken to document whether the genome was single or double stranded. The sensitivities of the various genomes to nuclease treatment were assessed by real-time RT-PCR, and digestion of the viral genome resulted in an increased Cp value (note the inverted y axis in Fig. 6) compared to the nondigested control. We included ISAV as a single-stranded RNA virus and IPNV as a positive control for a dsRNA virus. Nucleic acids isolated from the supernatants of cell cultures inoculated with PMCV, ISAV, and IPNV were subjected to enzymatic digestion to determine the character of the genome. Mung

FIG. 3. Negative staining of electron micrographs of virus isolated from heart tissue homogenate as described in Materials and Methods. Shown are virus-like particles of approximately 50 nm with regular surface structures (a) and possibly with surface projections (b). Bars = 40 nm (a) and 50 nm (b).

FIG. 4. Evolutionary relationships of members of the family *Totiviridae*. Phylogenetic analysis was based on a 480-aa sequence region encompassing the RdRp conserved motifs (right) of totiviruses, in line with the work published by Ghabrial and Nibert (13). Selected sequences were obtained from GenBank and aligned using the MUSCLE algorithm (version 3.8.31) with default settings (9). Neighbor-joining analysis was performed with 1,000 bootstrap replicates using Mega 4.0 software (36). Members of the family *Totiviridae* included in the tree are as follows: HvV190, *Helminthosporium victoriae* virus 190S (HVU41345); CeRV1, *Chalara elegans* RNA virus 1 (AY561500); CmRV, *Coniothyrium minitans* RNA virus (AF527633); EfV1, *Epichloe festucae* virus 1 (AM261427); GaRV-L1, *Gremmeniella abietina* RNA virus L1 (AF337175); HmTV1-17, *Helicobasidium mompa* totivirus 1 to 17 (AB085814); MoV1, *Magnaporthe oryzae* virus 1 (AB176964); SsRV1, *Sphaeropsis sapinea* RNA virus 1 (AF038665); SsRV2, *Sphaeropsis sapinea* RNA virus 2 (AF039080); BFTV1, *Botryotinia fuckeliana* totivirus 1 (NC_009224); MoV2, *Magnaporthe oryzae* virus 2 (AB300379); EbRV1, *Eimeria brunetti* RNA virus 1 (AF356189); LRV1-1, *Leishmania* RNA virus 1-1 (M92355); LRV2-1, *Leishmania* RNA virus 2-1 (U32108); LRV 1-4, *Leishmania* RNA virus 1-4 (U01899); TVV1, *Trichomonas vaginalis* virus 1 (U08999); TVV2, *Trichomonas vaginalis* virus 2 (AF127178); TVV3, *Trichomonas vaginalis* virus 3 (AF325840); ScV-L-A, *Saccharomyces cerevisiae* virus L-A (J04692); ScV-L-BC, *Saccharomyces cerevisiae* virus L-BC (U01060); UmV-H1, *Ustilago maydis* virus H1 (U01059); GLV, *Giardia lamblia* virus (L13218); IMNV, penaeid shrimp infectious myonecrosis virus (AY570982).

bean nuclease is a single-strand-specific DNA and RNA endonuclease, while RNase A catalyzes the degradation of RNA. RNase A digests both single-stranded and double-stranded RNA under low-salt conditions, while only single-stranded RNA is digested under high-salt conditions. As shown in Fig. 6, the genomes of PMCV and IPNV were sensitive to RNase A under low-salt conditions only and were reduced by 99.96 and 99.83%, respectively, and both were protected against RNase A digestion under high-salt conditions. IPNV and PMCV were also resistant to digestion by mung bean nuclease (Fig. 6). Single-stranded orthomyxovirus (ISAV) was sensitive to mung bean nuclease, with a reduction of 98.95% of the virus genome detected after enzyme digestion (Fig. 6). For RNase

FIG. 5. Genome organization of PMCV. The genome includes three large ORFs. The predicted ORF1 and ORF2 resemble two overlapping open reading frames. ORF2 is translated as a fusion protein with ORF1 due to ribosomal -1 frameshifting or as an individual protein (start codon indicated). ORF3 is a nonoverlapping frame.

A, there were reductions of 99.90% and 99.99% for high and low salt concentrations, respectively, after digestion (Fig. 6). None of the three viruses was shown to be digested using DNase I (results not shown), which was also verified by lack of a PCR product when the reverse transcription step was omitted prior to PCR. On this basis, we concluded that the PMCV genome was dsRNA.

Experimental infection of salmon with PMCV causes heart pathology typical of CMS. The next step was to confirm by experimental challenge that virus grown *in vitro* would result in CMS. Atlantic salmon without any prior disease history were injected with cell culture-derived challenge material passed through a 0.22- μ m filter, removing any bacteria present. Unchallenged fish were also added to the tank to investigate the transmissibility of the disease. Ten fish served as negative controls, and examination at the start of the experiment showed normal heart histology and absence of PMCV by real-time RT-PCR. Heart samples were collected postchallenge and analyzed for pathological changes, seen at 6 weeks postchallenge in the injected group, when 4 out of 6 fish exhibited various degrees of histomorphological changes ($x_{\text{avg}} = 0.87$) (Fig. 7a). By 7, 8, 9, and 10 weeks p.c., all fish (8/8) at all time points had myocyte degeneration and inflammatory changes in the spongy layer of the heart ventricle, changes typically seen in fish with CMS (Fig. 7b). The histoscores at different times postchallenge are shown in Fig. 8, showing that the maximum average score is seen at 9 weeks p.c. and declines slightly by 10 weeks p.c. (a nonsignificant reduction). In the cohobant group, fish were found to be positive, with typical CMS changes by 6 weeks, but with low scores ($x_{\text{avg}} = 0.22$), and 2 of 6 fish were found to have histological changes (3 of 6 had atrium present in sections). By 8 weeks, only 1 fish was found to be positive, and by 10 weeks postchallenge, x_{avg} was 0.29 (3 of 7 fish were positive). At both time points, the fish had lower scores than the injected group (Fig. 8).

The viral load of heart tissue coincides with the development of heart pathology. Quantitative analysis using real-time RT-PCR was performed to document the relationship between the histological score and the viral load in heart muscle following challenge with PMCV (Fig. 9). For Atlantic salmon challenged with GF-1-grown virus delivered by intramuscular injection, the viral load in heart muscle increased gradually and peaked 7 weeks postchallenge ($\sim 2,000$ -fold increase). PMCV virus was detected in 3 out of 6 sampled fish at 2 weeks p.c. The viral load, however, was very low in all positive fish at this time point, with a marked increase at 4 weeks p.c. (~ 35 -fold in-

FIG. 6. Sensitivities to nuclease digestion of three different RNA viruses were assessed by real-time PCR in order to determine the character of the PMCV genome. The following viruses were included: PMCV, IPNV (dsRNA), and ISAV (ssRNA). All treatments were performed in duplicate, and the results are given as Cp values, with ranges indicated by the error bars. Treatments were as follows: 1, untreated; 2, mung bean nuclease; 3, 2 µg RNase A, low-salt conditions; 4, 2 µg RNase A, high-salt conditions. All treatments were done in duplicate. RNase A treatment at low salt concentration produced reductions of 99.96 and 99.83% for PMCV and IPNV, respectively, while both were resistant to mung bean nuclease. ISAV genome levels were reduced by 98.95% following mung bean treatment and >99.91% following RNase A treatment.

crease). Viral levels thereafter reached a plateau between 6 and 10 weeks p.c., concurrent with the presence of histopathological changes typical of CMS.

Virus levels were examined by real-time RT-PCR in cohabitant fish to document horizontal transmission of infection (Fig. 9). In cohabitant fish, the presence of virus was first detected at 8 weeks p.c., with further increase at 10 weeks p.c. (~100-fold increase). The time lag between injected and cohabitant groups was approximately 4 weeks, indicating that there is a delay in shedding of infective virus particles from injected fish. Considerable variation between individual fish at the same sampling point was observed in all groups, likely explained by random events during sampling due to intraorgan variation of virus copy numbers (Fig. 9). All fish were also tested for PRV infection and were found negative (not shown).

Clinical CMS correlates with the presence of PMCV in heart tissue. To examine whether clinical CMS in farmed fish coincides with the presence of PMCV in heart tissue, samples were collected from three fish farms. Two of these (1 and 2) had a CMS diagnosis, while site three had no history of CMS. Fish collected from sample 1 (at slaughter) were found to have histomorphological changes typical of CMS, with scores ranging from 0.6 to 4 (covering the entire scale) (Fig. 10). The

relationship between histoscores and Cp values as assessed by real-time RT-PCR shows that there is a clear tendency for larger amounts of virus to be found in heart tissue with high histoscores (Fig. 10) ($r^2 = 0.76$). As it has been suggested that the biggest fish die most frequently from CMS, the Cp values were also plotted against the condition factor (weight $\times 100/\text{length}^3$) of the fish without any correlation being found ($r^2 = 0.08$).

For sample 2 (clinical CMS), 10 out of 10 fish were positive by real-time RT-PCR, and all showed histopathological changes typical of CMS. These fish were also tested for concomitant infection with PRV (27), and all fish showed variable levels of virus. For sample 3 (history of HSMI), no fish were found positive for PMCV by real-time RT-PCR, while all were found positive for PRV by real-time RT-PCR. None of the fish had changes typical of CMS, while 7/10 had histopathological changes of the heart ventricle indistinguishable from HSMI. These findings show that clinical cases of CMS correlate with the presence of PMCV in heart tissue and further substantiate that PMCV is the causative agent of CMS. Concurrent infection by PMCV and PRV may occur, and potential interactions between the two viruses for disease development and severity need to be elucidated.

FIG. 7. (a) Atrium at 8 weeks postchallenge. The foci show infiltration with lymphocytic cells (arrow) in proximity to a degenerate myofiber (asterisk) (H&E counterstain). (b) Ventricle at 8 weeks postchallenge. Shown is diffuse infiltration with inflammatory cells (lymphocytic cells and macrophages [arrow]) in and in proximity to a degenerate/necrotic myofiber (asterisk) (H&E counterstain).

FIG. 8. Histoscores for injection-challenged (Injected) and cohabitation-challenged (Cohabitants) salmon at different time postchallenge. No changes were observed by 4 weeks for the injected group, with gradual increases from 6 weeks up to 9 weeks. Fish challenged by cohabitation were collected at 6, 8, and 10 weeks postchallenge. Lower scores were found for this group, but distinct changes were present. Average values plus standard errors of the mean (SEM) are shown ($n = 6$ to 7 individuals per time point).

In situ hybridization shows localization of virus to degenerate/necrotic myocardial cells. We then went on to further document the association between myocardial changes and the presence of virus by using *in situ* hybridization. As can be seen in Fig. 11a, viral RNA can be detected in degenerate and necrotic cardiomyocytes, while no or very faint reaction was seen in normal cardiomyocytes. Localization of virus from *in situ* hybridization shows a diffuse presence in the sarcoplasm and also in small vesicular structures (Fig. 11b). Surrounding inflammatory cells or proliferating endothelial cells were negative (Fig. 11a and b). These findings clearly document that virus associates with degenerate/necrotic myocardial cells, indicating direct damage to the cells from viral replication.

DISCUSSION

The term CMS has been used to reflect a myocardial disease of unknown etiology. Here, we show that CMS of Atlantic

FIG. 9. Sequential development of the viral load in heart specimens following experimental challenge of Atlantic salmon. The viral load of PMCV was measured by real-time PCR. Data were normalized against endogenous β -actin, and virus levels are given as relative copy numbers (the fish with the lowest viral load was set to 1). The results are presented as a box plot (whiskers and minimum to maximum). Fish were challenged by intramuscular injection (Injected) or infected by cohabitation (Cohabitants; stippled boxes).

FIG. 10. Bivariate plot showing the relationship between the histoscore of the spongy part of the heart ventricle and the virus load (expressed as Cp values) in heart tissue for sample 1 ($n = 40$).

salmon is caused by a novel infectious agent, a dsRNA virus that is related to members of the family *Totiviridae*, genus *Giardiavirus*. As the virus causes a necrotizing myocarditis in salmon, the name piscine myocarditis virus is proposed. To our knowledge, PMCV is the first putative member of the family *Totiviridae* that infects a vertebrate host. Viruses of this family usually asymptotically and persistently infect fungi and protozoans, and most members of the family *Totiviridae* can be transmitted to new cells only during cell division, sporogenesis, and cell fusion. However, unlike other members of the *Totiviridae*, GLV can be shed extracellularly and is highly infectious in its purified form (23, 39).

The genomes of *Totiviridae* are composed of a single 4.6- to 6.7-kbp molecule of dsRNA containing two ORFs coding for a coat protein and an RdRp (<http://expasy.org/viralzone>). Although sequence-based comparison identifies GLV as the closest relative of PMCV, other properties of PMCV seem to better match those of IMNV of shrimp. Both PMCV and IMNV cause myonecrosis, and while IMNV causes skeletal muscle necrosis of shrimp (32, 37), PMCV infection results in cardiomyocyte necrosis and inflammation. Skeletal muscle cell pathology has not been observed following PMCV infection, whether experimental infections or clinical cases of CMS. IMNV, not yet assigned as a member of the family *Totiviridae*, differs from the family members with respect to a number of characteristics, such as infecting a host other than a fungus or a protozoan. The genome of IMNV is also larger than those of members of the family *Totiviridae* (7,560 bp) and encodes proteins other than the major coat protein (MCP) and RdRp, and like GLV, IMNV can be transmitted extracellularly (37), a trait shared with PMCV. Recently, two members of the family *Totiviridae* sharing similarities with IMNV have been isolated from arthropods, *Drosophila* totivirus (DTV) and *Armigeres subalbatus* totivirus (AsTV), infecting the common fruit fly and mosquitoes, respectively (40, 41). IMNV, AsTV, and DTV all have similar genome sizes and organizations and form a cluster distinct from other members of the family *Totiviridae*, and they have recently been proposed as members of a novel genus, "Artivirus" (derived from arthropod totivirus) (41). The organization of ORFs encoding the putative viral proteins in the genome of PMCV is similar to that of the family *Totiviridae*, but with one major difference: the third putative ORF identified downstream of the RdRp gene. While three putative ORFs have been described for other members of the family

FIG. 11. (a) *In situ* hybridization showing the presence of the viral genome in degenerate cardiomyocytes (reddish coloration). Note that endothelial cells are negative (arrows) (counterstaining with hematoxylin). (b) *In situ* hybridization showing the granular staining pattern (arrows) of the sarcoplasm in an area with degenerate cardiomyocytes. Note the enlarged nucleus (counterstaining with hematoxylin).

Totiviridae (35), PMCV seems to be the first member that encodes gene products from three ORFs. A third, small ORF is present in the 5' untranslated region (UTR) of *Leishmania* RNA virus 1 (LRV-1), but there are no reports that this small ORF encodes a gene product (35). Also, genome sequencing of *Chalara elegans* RNA virus 1 (CeRV1) revealed three putative ORFs, as a stop codon dividing the RdRp ORF into two halves was reported (28). However, this is likely due to sequencing errors (13).

IMNV of shrimp can be transmitted horizontally through the release of virus particles from infected hosts/cells, and similar mechanisms seem to be involved in PMCV infection. We base this interpretation on the fact that passage of supernatant from *in vitro*-grown PMCV results in CPE in infected GF-1 cell cultures. The CPE subsides with increasing passage number, indicating suboptimal growth conditions in the cell line. Intracellular virus replication was observed in CHH-1, with moderate release of virus into the extracellular compartment (Fig. 2a and b), but obvious CPE was not present. While the general understanding would be that naked viruses release their progeny through cell lysis (38), our findings support virus release occurring without CPE being found by visual inspection of the cell cultures. This observation is not unique, and release of another fish pathogenic virus, infectious pancreatic necrosis virus, into cell culture supernatants at high copy numbers without obvious CPE has been observed (19, 22). Further, release of rotavirus from polarized epithelial cells *in vitro* has also been observed to occur without cell lysis (17).

Our *in vivo* experiments also support horizontal transfer of virus. It was shown that salmon injected intramuscularly with PMCV transfer the virus through water to cohabitating fish that then develop heart changes typical of CMS. The histopathological changes of experimentally infected fish are similar to what has been described for challenge studies using organ homogenates from diseased fish (12) and similar to what is seen from clinical cases of CMS, as observed in this study. These observations indicate that PMCV spreads systemically from the site of injection to the preferred site, the heart, following intramuscular injection of virus. Further, PMCV is able to spread to the water and infect cohabitating fish, likely via

mucosal surfaces (gill, stomach, gut, or even skin), the details of which are not known and will be the subject of future studies. The primary replication site of PMCV remains to be identified, which is important for understanding the pathogenic sequence of CMS, and to understand the spread of the virus in fish populations, it will be important to document what role the mucosal surfaces play as a potential primary replication site. The findings made thus far clearly indicate extracellular spread of PMCV, showing similarities of infection patterns to those of IMNV and also GLV. It should also be added that a *Giardia*-like virus has been described from freshwater crayfish (*Cherax quadricarinatus*) from Australia (10). However, as no sequence information is available (10), it is difficult to ascertain if this is another putative member of the *Totiviridae* infecting crustaceans.

An infection mechanism including horizontal spread of PMCV and IMNV is an unusual trait among the members of the *Totiviridae*. For IMNV, studies using electron cryomicroscopy and three-dimensional (3D) image reconstruction showed that the virions have fiber-like protrusions (37), which is a novel feature of the family *Totiviridae*. The fiber-like protrusions are suggested to participate in the extracellular transmission of the virus and thus to play a role in viral pathogenesis (37). The molecular mass of one fiber complex of IMNV was estimated to be ~90 kDa, and the complex is formed as a trimer; thus, the predicted size of the peptide forming the protrusions was ~30 kDa. The predicted molecular mass of the ORF3-encoded protein is 33.4 kDa, and this protein remains a candidate for formation of surface structures of PMCV. Transmission electron microscopy using negative staining does not provide sufficient resolution to ascertain the presence of surface projections on PMCV, but formation of surface protrusions would align well with the observation that these structures are important for extracellular transmission.

A key question is how the totivirus ancestor acquired the ability to spread between cells, i.e., acquired the ability for horizontal spreading. A recent study of the structural traits of birnaviruses has pointed toward structural, functional, and possibly evolutionary links between positive-strand RNA and dsRNA viruses (6). The major capsid protein of the *Birnaviri-*

dae is the VP2 protein, which is an intermediate between the outer shell proteins of the dsRNA T = 13 *Reoviridae* and those of the positive-strand ssRNA T = 3 nodaviruses and T = 4 tetraviruses (24, 34). The birnavirus VP2 is built from a shell (S) as a base with projections (P) at the exterior. The S of VP2 is built from β -barrel strands and is tangential to the capsid surface, as in nodavirus/tetravirus, while the P domains are equivalent to the radially oriented β -barrels of *Reoviridae* capsid proteins (1). On this basis, Coulibaly et al. (6) proposed that a totivirus-like ancestor acquired its capsid from a birnavirus, which possibly would facilitate horizontal spread of the virus. The hypothesis also proposes that attaining such a trait would create the precursor of the *Reoviridae*.

Similarities between the genomes of viruses infecting hosts in different phyla or divisions are indicative of common ancestors and suggest that at some point, ancestral viruses switched between hosts that are distant from an evolutionary viewpoint (14) or that viruses switched/exchanged genome fragments. Nodaviruses infect finfish, including salmon (15), in the marine environment, as do *Birnaviridae* (infectious pancreatic necrosis virus infection of salmon). Recently a piscine reovirus was described as the likely cause of HSMI (27), and now we have indications of a representative of the *Totiviridae* infecting finfish. These are interesting findings in relation to virus evolution, and future studies should address the possible interactions between different virus species in the marine environment.

The ORF3-encoded protein showed no significant similarity with any other known proteins. An interesting observation is the presence of a putative chemokine superfamily motif in the N terminus of the ORF3 gene product. Chemokines are known to interact with a subset of 7-transmembrane, G protein-coupled receptors that are selectively found on the surfaces of their target cells (25). The use of chemokine receptors for cell entry is also found for human immunodeficiency virus (HIV). Infection is initiated by the external envelope glycoprotein, gp120, of HIV-1 and sequential interactions with two cellular receptors, CD4 and a chemokine receptor (or coreceptor) like CCR5 or CXCR4 (18, 21). To what extent PMCV uses specific chemokine receptors for cellular entry remains to be proven. Obviously, this remains speculative, and it will be necessary to document what role products of ORF3 would play in the infection pathogenesis, but an interesting observation is the localization of virus to the spongy structures of the heart, where the myocytes are covered with an endothelial lining and the only mode of infection of the myocytes would be through the endothelium. Yet another possibility is that the ORF3-encoded proteins play a role in enhancing or modulating the inflammation observed in CMS, which can be very prominent in chronic cases of the disease. The importance of ORF3 for viral pathogenesis and host responses awaits future research.

We performed a homology search with the predicted amino acid sequence of ORF1, but it produced no matches with significant similarity in GenBank. Nevertheless, the encoded protein of ORF1 from IMNV that is predicted to constitute the coat protein shares molecular mass and pI with the encoded protein of ORF1 of PMCV. The importance of these findings needs to be studied in more detail.

In this paper, we describe the causative agent of CMS as a double-stranded RNA virus with the suggested name piscine

myocarditis virus. Based on the RdRp sequence, PMCV clusters together with members of the family *Totiviridae*, genus *Giardiavirus*. PMCV also shares traits with IMNV, which is not yet assigned to the virus family. PMCV and IMNV have a totally different host range and putatively more complex coat structure that may be involved in transmissibility, which is not a common feature among members of the *Totiviridae*. Both viruses also cause clinical disease. Thus, further analysis will be required to determine the phylogenetic relationship of PMCV to the family *Totiviridae*.

ACKNOWLEDGMENTS

We thank Marianne Frøystad-Saugen (Pharmaq) for her contributions in the challenge trial and during initial experiments and discussions, Bjørn Midttun (Pharmaq) for help with the challenge experiment, and Svein Alexandersen (Pharmaq) for help with field sampling and enthusiasm to start the project. Jon Bohlin (Norwegian School of Veterinary Science) is thanked for his assistance with analysis and preparation of the phylogenetic trees. We are also grateful to Olav Breck and Eirik Hoel, Marine Harvest ASA, for the collection of samples from clinical case 1 of CMS and to Øyvind Kileng, Nofima Marine, for allowing us to use the ISA virus primers.

This work was supported by the Research Council of Norway, projects no. 190473 and no. 200511.

REFERENCES

- Ahlquist, P. 2005. Virus evolution: fitting lifestyles to a T. *Curr. Biol.* **15**: R465–R467.
- Amin, A. B., and J. Trasti. 1988. Endomyocarditis in Atlantic salmon in Norwegian sea farms—a case report. *Bull. Eur. Assoc. Fish Pathol.* **8**:70–71.
- Brocklebank, J., and S. Raverty. 2002. Sudden mortality caused by cardiac deformities following seining of preharvest farmed Atlantic salmon (*Salmo salar*) and by cardiomyopathy of postintra-peritoneally vaccinated Atlantic salmon parr in British Columbia. *Can. Vet. J.* **43**:129–130.
- Brun, E., T. Poppe, A. Skrudland, and J. Jarpe. 2003. Cardiomyopathy syndrome in farmed Atlantic salmon *Salmo salar*: occurrence and direct financial losses for Norwegian aquaculture. *Dis. Aquat. Organ.* **56**:241–247.
- Chi, S. C., W. W. Hu, and B. J. Lo. 1999. Establishment and characterization of a continuous cell line (GF-1) derived from grouper, *Epinephelus coioides* (Hamilton): a cell line susceptible to grouper nervous necrosis virus (GNNV). *J. Fish Dis.* **22**:173–182.
- Coulibaly, F., et al. 2005. The birnavirus crystal structure reveals structural relationships among icosahedral viruses. *Cell* **120**:761–772.
- Djikeng, A., et al. 2008. Viral genome sequencing by random priming methods. *BMC Genomics* **9**:5.
- Djikeng, A., and D. Spiro. 2009. Advancing full length genome sequencing for human RNA viral pathogens. *Future Virol.* **4**:47–53.
- Edgar, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**:1792–1797.
- Edgerton, B., L. Owens, B. Glasson, and S. Debeer. 1994. Description of a small dsRNA virus from fresh-water crayfish *Cherax quadricarinatus*. *Dis. Aquat. Organ.* **18**:63–69.
- Ferguson, H. W., T. Poppe, and D. J. Speare. 1990. Cardiomyopathy in farmed Norwegian salmon. *Dis. Aquat. Organ.* **8**:225–231.
- Fritsvold, C., et al. 2009. Experimental transmission of cardiomyopathy syndrome (CMS) in Atlantic salmon *Salmo salar*. *Dis. Aquat. Organ.* **87**: 225–234.
- Ghabrial, S. A., and M. L. Nibert. 2009. Victorivirus, a new genus of fungal viruses in the family Totiviridae. *Arch. Virol.* **154**:373–379.
- Gibbs, M. J., and G. F. Weiller. 1999. Evidence that a plant virus switched hosts to infect a vertebrate and then recombined with a vertebrate-infecting virus. *Proc. Natl. Acad. Sci. U. S. A.* **96**:8022–8027.
- Grotmol, S., G. K. Totland, and H. Kryvi. 1997. Detection of a nodavirus-like agent in heart tissue from reared Atlantic salmon *Salmo salar* suffering from cardiac myopathy syndrome (CMS). *Dis. Aquat. Organ.* **29**:79–84.
- Jacks, T., et al. 1988. Characterization of ribosomal frameshifting in HIV-1 gag-pol expression. *Nature* **331**:280–283.
- Jourdan, N., et al. 1997. Rotavirus is released from the apical surface of cultured human intestinal cells through nonconventional vesicular transport that bypasses the Golgi apparatus. *J. Virol.* **71**:8268–8278.
- Jülg, B., and F. D. Goebel. 2005. What's New in HIV/AIDS? Chemokine receptor antagonists: a new era of HIV therapy? *Infection* **33**:408–410.
- Kennedy, J. C., and R. D. Macdonald. 1982. Persistent infection with infectious pancreatic necrosis virus mediated by defective-interfering (DI) virus particles in a cell line showing strong interference but little DI replication. *J. Gen. Virol.* **58**:361–371.

20. Lannan, C. N., J. R. Winton, and J. L. Fryer. 1984. Fish cell lines: establishment and characterization of nine cell lines from salmonids. *In Vitro* **20**: 671–676.
21. Lusso, P. 2006. HIV and the chemokine system: 10 years later. *EMBO J.* **25**:447–456.
22. Marjara, I. S., B. J. Thu, and O. Evensen. 2010. Differentially expressed genes following persistent infection with infectious pancreatic necrosis virus in vitro and in vivo. *Fish Shellfish Immunol.* **28**:845–853.
23. Miller, R. L., A. L. Wang, and C. C. Wang. 1988. Identification of *Giardia lamblia* isolates susceptible and resistant to infection by the double-stranded RNA virus. *Exp. Parasitol.* **66**:118–123.
24. Munshi, S., et al. 1996. The 2.8 Å structure of a T = 4 animal virus and its implications for membrane translocation of RNA. *J. Mol. Biol.* **261**:1–10.
25. Murdoch, C., and A. Finn. 2000. Chemokine receptors and their role in inflammation and infectious diseases. *Blood* **95**:3032–3043.
26. Nibert, M. L. 2007. '2A-like' and 'shifty heptamer' motifs in penaeid shrimp infectious myonecrosis virus, a nonsegmented double-stranded RNA virus. *J. Gen. Virol.* **88**:1315–1318.
27. Palacios, G., et al. 2010. Heart and skeletal muscle inflammation of farmed salmon is associated with infection with a novel reovirus. *PLoS One* **5**:e11487.
28. Park, Y., D. James, and Z. K. Punja. 2005. Co-infection by two distinct totivirus-like double-stranded RNA elements in *Chalara elegans* (*Thielaviopsis basicola*). *Virus Res.* **109**:71–85.
29. Plant, E. P., and J. D. Dinman. 2006. Comparative study of the effects of heptameric slippery site composition on –1 frameshifting among different eukaryotic systems. *RNA* **12**:666–673.
30. Plant, E. P., et al. 2003. The 9-A solution: how mRNA pseudoknots promote efficient programmed –1 ribosomal frameshifting. *RNA* **9**:168–174.
31. Poppe, T. T., and S. L. Seierstad. 2003. First description of cardiomyopathy syndrome (CMS)-related lesions in wild Atlantic salmon *Salmo salar* in Norway. *Dis. Aquat. Organ.* **56**:87–88.
32. Poulos, B. T., K. F. Tang, C. R. Pantoja, J. R. Bonami, and D. V. Lightner. 2006. Purification and characterization of infectious myonecrosis virus of penaeid shrimp. *J. Gen. Virol.* **87**:987–996.
33. Rodger, H., and T. Turnbull. 2000. Cardiomyopathy syndrome in farmed Scottish salmon. *Vet. Rec.* **146**:500–501.
34. Schneemann, A., V. Reddy, and J. E. Johnson. 1998. The structure and function of nodavirus particles: a paradigm for understanding chemical biology. *Adv. Virus Res.* **50**:381–446.
35. Stuart, K. D., R. Weeks, L. Guilbride, and P. J. Myler. 1992. Molecular organization of *Leishmania* RNA virus 1. *Proc. Natl. Acad. Sci. U. S. A.* **89**:8596–8600.
36. Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24**:1596–1599.
37. Tang, J., et al. 2008. Infectious myonecrosis virus has a totivirus-like, 120-subunit capsid, but with fiber complexes at the fivefold axes. *Proc. Natl. Acad. Sci. U. S. A.* **105**:17526–17531.
38. Tucker, S. P., and R. W. Compans. 1993. Virus infection of polarized epithelial cells. *Adv. Virus Res.* **42**:187–247.
39. Wang, A. L., H. M. Yang, K. A. Shen, and C. C. Wang. 1993. *Giardiavirus* double-stranded RNA genome encodes a capsid polypeptide and a gag-pol-like fusion protein by a translation frameshift. *Proc. Natl. Acad. Sci. U. S. A.* **90**:8595–8599.
40. Wu, Q., et al. 2010. Virus discovery by deep sequencing and assembly of virus-derived small silencing RNAs. *Proc. Natl. Acad. Sci. U. S. A.* **107**:1606–1611.
41. Zhai, Y., et al. 2010. Isolation and full-length sequence analysis of *Armigeres subalbatus* totivirus, the first totivirus isolate from mosquitoes representing a proposed novel genus (*Artivirus*) of the family *Totiviridae*. *J. Gen. Virol.* **91**:2836–2845.