Check for updates

Received: 13 January 2020 | Revised: 4 March 2020 | Accepted: 5 March 2020

DOI: 10.1111/jfd.13166

SHORT COMMUNICATION

Journal of Fish Diseases WILEY

Lack of evidence of vertical transmission of piscine myocarditis virus in Atlantic salmon (*Salmo salar* L.)

Aase B. Mikalsen[1] | Morten Lund[2] | Farah Manji[3] | Mari Viken Kjønstad[3] | Per Helge Bergtun[3] | Gordon Ritchie[3] | Vidar Aspehaug[2] | Magnus Devold[2] | Øystein Evensen[1]

[1]Department of Basic Science and Aquatic Medicine, Faculty of Veterinary Medicine, Norwegian University of Life Science, Oslo, Norway

[2]PatoGen AS, Ålesund, Norway

[3]Mowi ASA, Bergen, Norway

Correspondence: Aase B. Mikalsen, Department of Basic Science and Aquatic Medicine, Faculty of Veterinary Medicine, Norwegian University of Life Science, Oslo, Norway.
Email: aase.mikalsen@nmbu.no

Keywords: Atlantic salmon, cardiomyopathy syndrome, piscine myocarditis virus, vertical transmission

1 | INTRODUCTION

Piscine myocarditis virus (PMCV) is the causal agent of cardiomyopathy syndrome (CMS), a disease affecting the heart of Atlantic salmon (Haugland et al., 2011). The disease has been found in Norwegian Atlantic salmon since 1988 (Amin & Trasti, 1988) and is primarily affecting adult Atlantic salmon 14–18 months after sea transfer and close to harvest, although recently outbreaks in young post-smolts have been reported (Svendsen et al., 2019; Wiik-Nielsen, Alarcon, Jensen, Haugland, & Mikalsen, 2016).

PMCV has been detected at a high prevalence in broodfish in freshwater, including high levels of virus-specific RNA, but with varying presence of CMS disease (Bang Jensen, Nylund, Svendsen, Ski, & Takle, 2019; Wiik-Nielsen, Ski, Aunsmo, & Lovoll, 2012) (personal observation/communication Per Helge Bergtun, Mowi and Even Thoen, PatoGen AS).

Horizontal transmission of virus and subsequent disease has been shown both in field studies and experimentally (Bang Jensen, Brun, Fineid, Larssen, & Kristoffersen, 2013; Fritsvold et al., 2009; Haugland et al., 2011). Two previous studies have also shown indications of vertical transmission of PMCV-specific RNA from broodfish to progeny, but the prevalence of positive individuals is low and CMS or other signs of disease have not been found in these individuals (Bang Jensen et al., 2019; Wiik-Nielsen, Ski, et al., 2012). Also, the virus has only been detected as virus-specific RNA using real-time PCR and at present there are no proofs that such detection of PMCV-specific RNA in progeny from PMCV-positive broodfish represents viable infectious virus particles causing CMS.

To add more results to the discussion on possible vertical transmission of PMCV in A. salmon and its relevance for causing CMS in later stages of the production cycle, we have performed two extensive studies where eggs from PMCV-positive females were fertilized with milt from PMCV-positive males. Fertilized eggs were split into two identical batches and shipped to one research facility in Norway and one in Canada for hatching and longitudinal screening. All life stages were screened using real-time PCR specific for PMCV to verify the presence of PMCV.

1.1 | Broodfish and fertilization of eggs

Broodfish from two commercial sites (A and B) were used for this study. Two groups of fertilized eggs were produced at Broodfish site A (groups A1 and A2), and group A2 was further split into two subgroups (A2a and A2b) according to conditions used for disinfection after fertilization. One group was produced at Broodfish site B (group B1). Both the female and male broodfish had been screened for PMCV-specific RNA in heart and in ovarian fluid and milt, respectively, prior to fertilization of eggs. Autopsy was performed by fish health personnel on the broodfish without resulting observations

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
© 2020 The Authors. *Journal of Fish Diseases* published by John Wiley & Sons Ltd

of clinical signs of CMS. An overview of broodfish, PMCV-specific RNA levels and fertilization groups is given in Table 1. Before fertilization, the eggs were rinsed four times with saline water (10.5–11.5‰). After fertilization, the eggs were rinsed with saline water, disinfected by incubation for 10 min in Buffodine according to the manufacturer's protocol and then rinsed in freshwater.

The fertilized eggs from all four groups were disinfected again, before shipping in identical replicates on wet ice by air transport to two aquaculture research facilities. At arrival at each of the two facilities, the eggs were disinfected after internal protocols and regulations for receiving external eggs (Table 2).

1.2 | Hatching and on-growing

The replicates of hatching and on-growing were performed under controlled clean environments in approved research facilities in Norway and Canada according to respective national legislations, and the trials were evaluated by ethical committee and approved according to fish welfare.

Replicate 1 was conducted at Havbruksstasjonen (Tromsø, Norway). Replicate 2 was conducted at Centre for Aquaculture Technologies (Prince Edwards Island, Canada). Hatching and start-feeding were performed according to normal procedures. The fingerlings were kept in flow-through systems with routine oxygen monitoring and were fed a standard commercial feed. The temperature was kept at 6–10 and 8.5–12°C, respectively, for the replicates 1 and 2 until termination of the trial.

1.3 | Sampling and preservation

Before shipment to the research facilities, 60 eggs were sampled from groups A1, A2a and A2b and 62 eggs from group B1 and subsequently subjected to RNA extraction and real-time PCR (Table 2). Similarly, 60 eggs were sampled from all groups after disinfection at arrival at facility 1. The eggs were sampled individually without preservation and shipped at 6°C for real-time PCR analysis.

From both facilities, samples from 30 to 60 individuals were collected at first feeders and at 2, 5, 10, 45 and 80 grams (Table 2). Tissue samples were taken as V-sections including heart and head kidney from the smallest life stages and subsequently from heart only. At sampling from 10-g fish at facility 1, head kidney was also included. All samples were preserved in RNA*later*™ (Ambion Inc.) and stored at 6°C for 24 hr, with subsequent transfer to −20°C.

1.4 | RNA extraction and real-time PCR

PatoGen AS (Norway) performed RNA extraction and real-time PCR analysis for PMCV RNA in all samples. The real-time PCR assay targeting PMCV is validated to ISO17025 standards by PatoGen AS. Samples were defined as positive when having a PMCV C_t lower than 37.0. Elongation factor 1α served as an internal reference gene (Lovoll, Austbo, Jorgensen, Rimstad, & Frost, 2011).

2 | RESULTS AND CONCLUSIONS

PMCV-specific RNA was detected in only one of a total of 482 fertilized eggs, at a C_t of 24.0. Also, no PMCV-specific RNA was detected in heart in any of the six life stages of offspring, tested between first feeders and 80 g smolt in a total of 2,417 individuals (Table 2). Since previous studies have indicated that PMCV might be present in high concentrations in kidney in early infection phases (Garseth, Fritsvold, Svendsen, Bang Jensen, & Mikalsen, 2018; Timmerhaus et al., 2011), we included PMCV RNA screening on kidney samples in addition to heart from all 10-g fish at facility 1. All kidney samples tested were found to be PMCV negative.

The positive egg was detected as one of 62 eggs examined in group B1 originating from parents with low PMCV RNA levels in male (negative milt/C_t 36.0 heart) combined with high levels in female (C_t 26.9 ovarian fluid/25.0 heart), giving a prevalence of 1.6%. Such a low level and negative results for the other three life stages make it difficult to conclude statistically that real-time PCR positivity (in one egg) is linked to (relatively) high levels of viral genome in the female parent. The sampling was performed after two disinfection rounds of the eggs before shipment to the research facilities. As for all batches, batch B1 was subsequently sent as in parallel to two facilities for hatching and on-growth. On arrival at facility 1, after a round of disinfection, 60 eggs from this batch were tested again and were all negative. After hatching, 30–60 samples from each facility

TABLE 1 Overview of groups of fertilized eggs and levels of PMCV-specific RNA in parental origin

		PMCV RNA levels (C_t values)						
		Male		Female		Disinfection procedure		
Broodfish site	Group	Milt	Heart	Ovarian fluid	Heart	After fertilization	Before shipment	At arrival in research facility
A	A1	Negative	22.8	35.8	32.0	Yes	Yes	Yes
	A2a	Negative	33.7	32.5	33.7	No	Yes	Yes
	A2b	Negative	33.7	32.5	33.7	Yes	Yes	Yes
B	B1	Negative	36.0	26.9	25.0	Yes	Yes	Yes

TABLE 2 Overview of handling and testing of eggs, fry and parr in facility 1 and number of samples with PMCV RNA-positive result. Similar handling and testing are valid for facility 2 with exceptions as commented, and all PCR test showed PMCV RNA-negative results

	PMCV RNA-positive individuals			
Handling and testing	**A1**	**A2a**	**A2b**	**B1**
Rinsing of eggs				
Fertilization				
Disinfection after fertilization				
Disinfection before shipment				
PCR test eggs before shipment	0/60	0/60	0/60	1/62
Shipment to aquaculture research facility				
Disinfection at arrival				
PCR test eggs after arrival disinfection[a]	0/60	0/60	0/60	0/60
Hatching				
PCR test first feeders[b]	0/60	0/60	0/60	0/60
PCR test 2 g[b]	0/60	0/60	0/60	0/60
PCR test 5 g[b]	0/60	0/60	0/60	0/60
PCR test 10 g[c]	0/60	0/60	0/60	0/59
PCR test 45 g[d]	0/60	0/60	0/60	0/60
PCR test 80 g[d]	0/60	0/60	0/60	0/60

[a]Not tested at facility 2.
[b]PCR test on 30 individuals from facility 2.
[c]Included kidney samples in addition to heart.
[d]PCR test on 30–60 individuals from facility 2.

were tested at six life stages up to 80 g, in total 628 individuals, all negative. On this basis, we conclude that PMCV was not transmitted vertically from parents to post-hatch offspring under these controlled study conditions.

The lack of detection of PMCV-specific RNA in our study is contrary to findings in the two previous similar studies (Bang Jensen et al., 2019; Wiik-Nielsen, Ski, et al., 2012). One major difference between the present study and the two previously published is the use of disinfection protocol. We used three rounds of disinfection including several steps of rinsing, and the fertilized eggs were tested after the second and third rounds of disinfection. Wiik-Nielsen et al. screened eggs before any disinfection with resulting PMCV RNA detection prevalence of 10%. Eggs were disinfected only once before hatching, and prevalence of PMCV-specific RNA on newly hatched fry was 25%, but no PMCV-specific RNA was detected at first feeding (Wiik-Nielsen, Ski, et al., 2012). Bang Jensen and co-workers also included one round of disinfection of eggs and screened for PMCV RNA before and after disinfection. This showed a clear reducing effect of disinfection on the prevalence of eggs with detectable PMCV RNA, including 8 of 14 egg batches where the disinfection procedure completely removed the virus. The batches with highest prevalence after disinfection were used for hatching and resulted in detected PMCV RNA at a low prevalence up to 70 g presmolt. The differences in disinfection regime and prevalence of detected PMCV-specific RNA in eggs and offspring in these three studies show that repeated rounds of disinfection can reduce PMCV-specific RNA contamination of fertilized eggs and offspring to zero. This is supported by the mentioned specific study of effect of disinfection on the prevalence of PMCV RNA on eggs from Bang Jensen and co-workers (Bang Jensen et al., 2019). Use of systematic measures to avoid spread of infectious agents and disinfection of eggs after fertilization particularly comply with current Norwegian regulations (Lovdata, 2019). The general practice in the egg production industry is disinfection after fertilization, before transfer to and at arrival to the hatchery, although disinfection at arrival might vary between producers. Rinsing of eggs four times in saline water before fertilization is not, as far as we know, common practice in the industry, although it is used by Mowi Norway as a part of the standard protocol. The effect of rinsing and disinfection procedures resulting in reduction of detectable PMCV RNA also indicates that eggs found to be positive for PMCV are contaminated by the virus on the outside of the egg and the virus is not in the internal parts of the egg or intracellular.

The C_t values of all positive samples from offspring in all studies show almost undetectable levels of PMCV-specific RNA, as the C_t values are above repeatable C_t values with the exception of one 15 g fry in the Bang Jensen et al. study showing at C_t of 33.9. Also, the studies indicate that the prevalence in offspring with detection of PMCV RNA is decreasing through the progressive life stages and eventually seems to disappear. It also has to be noted that real-time PCR analyses only detect a very limited region of a pathogens full genome and the detection could be remnants of non-viable degraded virus particles or particles with defect genomes. A study including infection of either naïve salmon or cells in culture is needed to document and conclude on the infectivity and virulence of viral particles related to the PMCV-specific RNA detected in the eggs/offspring in the studies. Studies on vertical transmission should also include sequencing of PCR products from parents and eggs/offspring followed by phylogenetic analyses/sequence alignments. Identical sequences would aid in interpretation of source. That said, previous and ongoing studies have shown that PMCV exists as quasispecies in infected salmon populations and even in single individuals, and minute nucleotide position differences in the genome have also been found. This, in turn, reduces the value of phylogenetic analyses in terms of back-tracking the source of infection in offspring (Wiik-Nielsen, Alarcon, Fineid, Rode, & Haugland, 2012, pers. obs. Aase B. Mikalsen).

In summary, we find that an extended disinfection regime is important for reduction of PMCV RNA contamination on eggs and subsequent transfer to offspring. It also points towards vertical transmission not being an important route for virus spread. This is supported by the fact that PMCV has not been introduced to Chile despite large-scale import of eggs from Norway, as shown by PMCV screening of Atlantic salmon with no positive samples since it started in 2013 (Lara, 2014).

ACKNOWLEDGEMENTS

The authors would like to acknowledge personnel at NOFIMA Tromsø and CATC for supervising the trials and for performing the samplings at the two facilities.

CONFLICT OF INTEREST

ML, MD and VA are affiliated by the diagnostic service company PatoGen AS, which offers PMCV screening.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

ORCID

Aase B. Mikalsen https://orcid.org/0000-0001-6367-9629

REFERENCES

Amin, A. B., & Trasti, J. (1988). Endomyocarditis in Atlantic salmon in Norwegian seafarms. *Bulletin European Association of Fish Pathologists*, *8*, 70–71.

Bang Jensen, B., Brun, E., Fineid, B., Larssen, R. B., & Kristoffersen, A. B. (2013). Risk factors for cardiomyopathy syndrome (CMS) in Norwegian salmon farming. *Diseases of Aquatic Organisms*, *107*, 141–150.

Bang Jensen, B., Nylund, S., Svendsen, J. C., Ski, P. R., & Takle, H. (2019). Indications for a vertical transmission pathway of piscine myocarditis virus in Atlantic salmon (Salmo salar L.). *Journal of Fish Diseases*, *42*, 825–833.

Lovdata. (2019). *Forskrift om drift av akvakulturanlegg (akvakulturdrifts-forskriften) FOR-2008-06-17-822*. Oslo, Norway: Ministry of Trade, Industry and Fisheries. Retrieved from https://lovdata.no/dokument/SF/forskrift/2008-06-17-822?q=Akvakulturdriftsforskriften

Fritsvold, C., Kongtorp, R. T., Taksdal, T., Orpetveit, I., Heum, M., & Poppe, T. T. (2009). Experimental transmission of cardiomyopathy syndrome (CMS) in Atlantic salmon Salmo salar. *Diseases of Aquatic Organisms*, *87*, 225–234.

Garseth, A. H., Fritsvold, C., Svendsen, J. C., Bang Jensen, B., & Mikalsen, A. B. (2018). Cardiomyopathy syndrome in Atlantic salmon Salmo salar L.: A review of the current state of knowledge. *Journal of Fish Diseases*, *41*, 11–26.

Haugland, Ø., Mikalsen, A. B., Nilsen, P., Lindmo, K., Thu, B. J., Eliassen, T. M., … Evensen, Ø. (2011). Cardiomyopathy syndrome of Atlantic salmon (*Salmo salar* L.) is caused by a dsRNA virus of the *Totiviridae* family. *Journal of Virology*, *85*, 5275–5286.

Lara, M. (2014). *Alphavirus, PRV and PMCV situation in Chile*. In: TriNation meeting, Trondheim, Norway.

Lovoll, M., Austbo, L., Jorgensen, J. B., Rimstad, E., & Frost, P. (2011). Transcription of reference genes used for quantitative RT-PCR in Atlantic salmon is affected by viral infection. *Veterinary Research*, *42*, 8.

Svendsen, J. C., Nylund, S., Kristoffersen, A. B., Takle, H., Fossberg Buhaug, J., & Jensen, B. B. (2019). Monitoring infection with Piscine myocarditis virus and development of cardiomyopathy syndrome in farmed Atlantic salmon (Salmo salar L.) in Norway. *Journal of Fish Diseases*, *42*(4), 511–518.

Timmerhaus, G., Krasnov, A., Nilsen, P., Alarcon, M., Afanasyev, S., Rode, M., … Jorgensen, S. M. (2011). Transcriptome profiling of immune responses to cardiomyopathy syndrome (CMS) in Atlantic salmon. *BMC Genomics*, *12*, 459.

Wiik-Nielsen, C. R., Ski, P. M., Aunsmo, A., & Lovoll, M. (2012). Prevalence of viral RNA from piscine reovirus and piscine myocarditis virus in Atlantic salmon, *Salmo salar* L., broodfish and progeny. *Journal of Fish Diseases*, *35*, 169–171.

Wiik-Nielsen, J., Alarcon, M., Fineid, B., Rode, M., & Haugland, O. (2012). Genetic variation in Norwegian piscine myocarditis virus in Atlantic salmon, Salmo salar L. *Journal of Fish Diseases*, *36*(2), 129–139.

Wiik-Nielsen, J., Alarcon, M., Jensen, B. B., Haugland, O., & Mikalsen, A. B. (2016). Viral co-infections in farmed Atlantic salmon, Salmo salar L., displaying myocarditis. *Journal of Fish Diseases*, *39*, 1495–1507.

How to cite this article: Mikalsen AB, Lund M, Manji F, et al. Lack of evidence of vertical transmission of piscine myocarditis virus in Atlantic salmon (*Salmo salar* L.). *J Fish Dis*. 2020;43:715–718. https://doi.org/10.1111/jfd.13166