ORIGINAL ARTICLE

Inactivation of *Piscine orthoreovirus*

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Funding information
Norwegian Seafood Research Fund, Grant/Award Number: 901305

Abstract

Piscine orthoreovirus infects various salmonid fish species, and the infection is associated with diseases such as heart and skeletal muscle inflammation (HSMI) in farmed Atlantic salmon (*Salmo salar*). There are no vaccines available or genetically selected resistant hosts that can efficiently control piscine orthoreovirus (PRV) infection. Currently, the only prophylactic measure against PRV is general biosecurity measures aiming to break the transmission cycle. Methods to eradicate infectious virus from contaminated facilities are desirable, but the knowledge on how to inactivate PRV is lacking. A major bottleneck for inactivation studies is the lack of ability to propagate PRV in cell culture. Therefore, in this study we developed an in vivo model for detection of infectious PRV particles after treatment of the virus with inactivation tools such as heat, pH, iodine, UV and commercially available disinfectants. The results show that standard iodine treatment is efficient in inactivation of the virus, and similarly are high and low pH extremes and treatment with Virocid, a commercially available disinfectant. A UV dose of at least 50 mJ/cm^2 is required for inactivation, and the virus has high resistance against heat treatment.

KEYWORDS

inactivation, iodine, *Piscine orthoreovirus*, UV, Virocid

1 | INTRODUCTION

There is a lack of efficient vaccines for many viral diseases in salmonid aquaculture which leaves zoo-sanitary measures as the major tool when trying to control the infections. The effect of such measures depends on basic knowledge of the infectious agents, their distribution and possible containment. *Piscine orthoreovirus* is a widespread agent in salmonids, but the lack of physical and chemical characterization of the virus prevents optimized biosecurity measures to control infection and associated disease.

Piscine orthoreovirus belongs to the family Reoviridae, genus *Orthoreovirus* (Markussen et al., 2013; Palacios et al., 2010). The

three subtypes of piscine orthoreovirus (PRV) that have been described, that is PRV-1 (Palacios et al., 2010), PRV-2 (Takano et al., 2016) and PRV-3 (Olsen, Hjortaas, Tengs, Hellberg, & Johansen, 2015), infect different salmonid species in which the outcome can be non-pathogenic or pathogenic (Di Cicco et al., 2017, 2018; Godoy et al., 2016; Hauge et al., 2017; Marty, Morrison, Bidulka, Joseph, & Siah, 2014; Polinski, Marty, Snyman, & Garver, 2019). Most studied is PRV-1 infection of Atlantic salmon (*Salmo salar*), where the virus can cause heart and skeletal muscle inflammation (HSMI), a common disease in farmed Atlantic salmon (Kongtorp, Kjerstad, Taksdal, Guttvik, & Falk, 2004; Wessel et al., 2017). PRV is widespread in salmonid aquaculture,

ubiquitous in the seawater grow-out phase, and also commonly found in presmolt facilities (Løvoll et al., 2012). Currently, there is no available vaccine against PRV infection. Consequently, prophylactic intervention against PRV and HSMI, if used at all, is based on implementation of biosecurity measures like screening of eggs and broodfish and attempts to eradicate the virus from presmolt facilities. However, lack of key information on the physical and chemical stability of the virus reduces the efficiency of PRV control measures. Since PRV has resisted propagation in cell lines, there is no specific information currently available for the effect of disinfection on PRV infectivity. However, the widespread distribution and common occurrence of PRV in Atlantic salmon farming indicate that the current risk-reducing measures are not efficient.

Like all orthoreoviruses, PRV has a segmented double-stranded RNA genome encompassed in a capsid composed of two protein layers (Key, Read, Nibert, & Duncan, 2013; Markussen et al., 2013). There is no lipid bilayer. Orthoreoviruses are known to be stable in the environment and not easy to inactivate. For example, the type species of orthoreovirus, mammalian reovirus (MRV), withstands 55°C, pH between 2 and 9, lipid solvents and detergents (Attoui et al., 2012). In the salmon aquaculture industry, many strategies are used to mitigate the risk of introduction of infectious agents, including UV treatment of the inlet water of presmolt facilities (Liltved, Vogelsang, Modahl, & Dannevig, 2006), iodine treatment of fertilized eggs (De Swaef, Van den Broeck, Dierckens, & Decostere, 2016), washing and disinfection of equipment, boats, fallowing, separation of generations, distance and contact between farms (Lillehaug, 2015). The effect of such measures will vary between the various infectious agents.

In general, studies of inactivation of virus infectivity measure reduction of titre of infectious virus over time during a specific treatment. This requires a system to measure infectivity and virus replication and quantify infectious virus (Verner – Jeffreys et al., 2009). Classically, this is done using a susceptible cell culture. In fish virology, the *infectious pancreatic necrosis virus* (IPNV) has often been used as a viral reference agent in Atlantic salmon aquaculture when setting up biosecurity plans, due to ease of cultivation in cell cultures and environmental stability. To overcome the methodical bottleneck of PRV resistance to propagate in cell culture, we aimed to develop an in vivo challenge model to study PRV inactivation. We recently developed a method to purify PRV particles from blood of infected fish and thus an alternative source to obtain pure and quantifiable batches of virus particles (Wessel et al., 2017).

In the present study, we characterized the effect of various inactivation procedures on purified and quantified PRV particles, and assessed infectivity in an in vivo challenge model, using individual fish as the virus replication entity. This model was used to investigate PRV resistance to heat, pH, UV, iodine, as well as the commercially available disinfectant Virocid, which is based on different types of quaternary ammonium, glutaraldehyde and isopropanol. The study provided novel information about PRV and an alternative model that potentially could be used in similar studies of other non-cultivable aquaculture viruses.

2 | MATERIALS AND METHODS

2.1 | Virus preparations

The PRV-1 NOR2012 strain used in the present study originated from a field outbreak of HSMI in Norway in 2012 (Lund et al., 2016) and has been characterized and sequenced earlier (Wessel et al., 2017). Purified PRV-1 NOR2012 was prepared from stored blood pellets with high PRV-1 content using a protocol described previously (Wessel et al., 2017). Briefly, blood pellets were diluted 1:10 in Leibovitz's L15 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and sonicated five times at 20 kHz for 30 s with 1-min rest in between. To a 5 ml sample, addition of 100 µL 10% (w/v) sodium desoxycholate (DOC) was followed by 5-s vortexing, 5-min rest, 5-s vortexing and 25-min rest; addition of 2 ml Vertrel XF (DuPont, Wilmington, DE, USA) was followed by 5-s vortexing and sonication at 20 kHz for 30 s; and another addition of 2 ml of Vertrel XF was followed by vortexing and sonication (as above), and then centrifuged at 9,000 × g for 10 min at 4°C. The aqueous phase was collected, and 4.5 ml Vertrel XF was added, vortexed, sonicated and centrifuged as above. Again, the top aqueous phase was collected and layered onto a caesium chloride gradient (1.22–1.45 g/ml) and centrifuged for >12 hr at 100,000 × g. The tube was punctured with a syringe to collect the visible virus band, and the collected material was dialysed using Slide-A-Lyzer Dialysis Cassette (Thermo Fisher Scientific), against three volumes of Dulbecco's PBS without calcium and magnesium, DPBS (Sigma-Aldrich, St. Louis, MO, USA) for >1 hr, >3 hr and then >12 hr. Viral particles were confirmed by inspection by transmission electron microscopy of negatively stained samples as previously described (Wessel et al., 2017). Glycerol was added to a final volume of 15% and the batch stored at −80°C until used.

To standardize the viral load, the copy number of PRV-1 particles in the purified samples was determined by RT-qPCR. In brief, RNA was isolated from 10 µl purified PRV-1 diluted in 130 µl DPBS using the QIAamp Viral RNA Kit (Qiagen) according to manufacturer's instructions, and RNA was eluted in 50 µl. The Qiagen OneStep Kit (Qiagen) was used for RT-qPCR for PRV-1 using 5 µl eluted RNA as input. Prior to RT-qPCR, the template RNA was denatured at 95°C for 5 min. The RT-qPCR targeted PRV-1 segment S1, and previously described conditions were used (Wessel, Olsen, Rimstad, & Dahle, 2015). The samples were run in triplicates and absolute quantification of PRV-1 particles was found by comparison to a standard curve made from in vitro transcripts from the PRV-1 S1 genomic segment, prepared as previously described (Wessel et al., 2017). A 10-fold serial dilution of transcripts (10^8 to 10^2 transcripts per sample) was run on the same RT-qPCR plate to determine the copy number of the batch. Primers and probe are listed in Table 1.

2.2 | Experimental fish

A total of 915 Atlantic salmon parr (initial weight 5 grams) of StofnFiskur Optimal strain were used, including one pilot study and

TABLE 1 Primers and probe

Primer/probes	Primer sequence (5' → 3')
σ3 S1–659 Fwd	TGCGTCCTGCGTATGGCACC
σ3 S1–801 Rev	GGCTGGCATGCCCGAATAGCA
σ3 S1–693 Probe	*FAM*-ATCACAACGCCTACCT–*MGBNFQ*

Note: The quencher and reporter of the probe are in italic. The GenBank accession number for the PRV-1 S1 is GU994022.

three inactivation studies (described in detail below). All studies had been approved by the Norwegian Food Safety Authority (NFDA) according to the European Union Directive 2010/63/EU for animal experiments (permit number 11,251). The challenge trials were conducted at VESO Vikan aquatic research facility (Namsos, Norway). Before initiation of the study, ten fish were screened and found negative for ISAV, SAV, PRV-1, PMCV and IPNV by qPCR. Prior to challenge, the fish were acclimatized for one week, fed according to standard procedures and kept under 24-hr light regime during the trials. The fish were kept in tanks supplied with freshwater at $12 \pm 1°C$ with continuous light and observed minimum once per day. The fish were anaesthetized by bath immersion in benzocaine chloride (2 ± 5 min, 0.5 g/10 L water) prior to handling and killed using a higher concentration of benzocaine chloride (1 g/5 L water).

2.3 | In vivo challenge model—Pilot study

A pilot trial with 105 fish was performed to optimize challenge dose and sampling time points and determine target organ for PRV-1 detection. Prior to challenge, five fish were sampled to confirm that the fish population was PRV-1-negative. The remaining fish were divided into five groups (20 fish each) and challenged by intramuscular (i.m.) injection (0.05 ml per fish) containing (1) high-dose PRV-1 (10^8 copies/fish), (2) medium-dose PRV-1 (10^6 copies/fish), (3) low-dose PRV-1 (10^4 copies/fish), (4) PBS or (5) high-dose PRV-1 (10^8 copies/fish), which had been heat-inactivated at 85°C for 25 min prior to injection (heat inactivation details described later). At 2, 4, 6 and 8 weeks post-challenge (wpc), five fish were sampled from each group, and spleen and head kidney were collected on RNAlater.

Total RNA was isolated from spleen and head kidney samples. The tissue samples were homogenized in QIAzol Lysis Reagent (Qiagen) using 5-mm steel beads and TissueLyser II (Qiagen) for 2×5 min at 25 Hz, and chloroform was added and centrifuged. The aqueous phase was collected and proceeded with automated RNA isolation using RNeasy Mini QIAcube Kit (Qiagen) as described by the manufacturer. The RNA was quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). The Qiagen OneStep Kit (Qiagen) was used for RT-qPCR for PRV-1 using 100 ng (5 μL of 20 ng/μL) of total RNA as input 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 run for 40 cycles using previously described conditions (Wessel et al., 2015). The samples were run in duplicate. Primers and probe are listed in Table 1.

2.4 | In vivo challenge model—Inactivation studies

A standardized model for inactivation studies was established based on the results from the pilot trial. In this model, purified PRV-1 was treated by different inactivation methods at a final concentration of 2.0×10^8 copies/ml. To control for batch-to-batch variation, each inactivation was performed using two batches of purified virus (Batch#1, Batch#2). Details of the specific inactivation methods are listed below and included heat, pH, UV, iodine and Virocid. To test the effect of the inactivation, 30 fish were injected i.m. with the treated virus (1.0×10^7 copies/fish), injecting 15 fish with each batch (Batch#1 unmarked and Batch#2 marked by adipose fin clipping). At 4, 6 and 8 wpc, spleen was sampled on RNAlater from ten fish, that is five from Batch#1 and five from Batch#2, for detection of PRV-1 by RT-qPCR. Control groups were included in the study using identical set-up and sampling, and details for each treatment and controls are described together. A total of three trials were performed in which different inactivation methods were tested. A complete overview of the trials, groups, controls, fish number and samplings is listed as supplementary data (Table S1).

2.5 | Detection of PRV-1 by RT-qPCR

Total RNA was isolated from spleen using RNeasy Mini QIAcube Kit (Qiagen) as described by the manufacturer. The RNA was quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). In analysis of trials I and II, the RT-qPCR for PRV-1 was performed using Qiagen OneStep Kit (Qiagen) and Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies) (Trial III) was used in trial III. An input of 100 ng (5 μl of 20 ng/μl) was used per reaction, and prior to RT-qPCR, the template RNA was denatured at 95°C for 5 min. The RT-qPCR targeted PRV-1 segment S1 and was performed using previously described conditions (Wessel et al., 2015). The samples were run in duplicates, and a sample was defined as positive if both parallel samples had a Ct < 35. Primers and probe are listed in Table 1.

2.6 | Temperature

Purified PRV-1 at a concentration of 2.0×10^9 copies/ml was diluted 1:10 in DPBS preheated to the correct temperature (final viral concentration of 2.0×10^8 copies/ml), kept at 85°C for 25 min, or 76°C for 20 min, or 56°C for 30 min, on a ThermoMixer compact (Eppendorf, Hamburg, Germany) with shaking (800 rpm) and then placed on ice. Fish were injected i.m. with 0.05 ml (1.0×10^7 copies per fish). The positive control was untreated purified virus of the same batch with the same concentration.

In addition to purified PRV-1, salmon blood containing PRV-1 was also heat-treated at 85°C for 25 min or 76°C for 20 min. In brief, PRV-1-containing blood pellet (Ct-value 15.4) was first diluted 1:3 in Leibovitz's L15 medium (Gibco) supplemented with gentamicin

at 50 µg/ml (Gibco,) to make a blood suspension. Thereafter, the blood suspension was diluted 1:10 in preheated L15 medium, incubated at the desired temperature for the set amount of time and placed on ice. Each fish was injected with 0.05 ml i.m. The positive control was the same diluted blood suspension but without heat treatment.

2.7 | pH

Purified PRV-1 at a concentration of 2.0×10^9 copies/ml was diluted 1:5 in DPBS adjusted to a final pH of 3 by addition of concentrated HCl, exposed for 30 min at room temperature and then neutralized by diluting 1:2 in DPBS with added concentrated NaOH to produce a final pH of 7.2. The final viral concentration was 2.0×10^8 copies/ml, and fish were injected with 0.05 ml (1.0×10^7 copies per fish).

Similarly, purified PRV-1 diluted 1:5 in DPBS adjusted to pH 12 by addition of concentrated NaOH, was exposed for 30 min at room temperature and then neutralized by diluting 1:2 in DPBS with added concentrated HCl to produce a final pH of 7.2. The final viral concentration, injection of fish and positive control were as described above.

2.8 | UV irradiation

An aliquot of 20 ml purified PRV-1 in DPBS at a concentration of 1.0×10^8 copies/ml was added to glass Petri dishes with a diameter of 47 mm. The transmissivity of suspension was 98.4%/cm. The dishes were UV-irradiated with 25, 50, 100, 200 and 300 mJ/cm^2 using a Collimated Beam Device, manufactured at WEDECO. The UV irradiation dose was calculated according to the UV dose equation given in the UVDGM Manual, Appendix C, C.2.4, Equation C.1 (USEPA, 2006). Fish were injected with 0.1 ml of irradiated samples (1.0×10^7 copies per fish), and positive control samples consisted of non-irradiated virus of the same batch and concentration.

2.9 | Iodine treatment

PRV-1 at a concentration of 2.0×10^9 copies/ml was diluted 1:1 in either 4%, 2% or 1% iodine (Buffodine, FishTech, Vestby, Norway) diluted in DPBS, for a final iodine concentration of 2%, 1% or 0.5% (200 ppm, 100 ppm and 50 ppm), and incubated for 10 min at 10°C. For the 1% concentration, incubation at 5 and 20 min was also tested. After incubation, the treated virus was diluted 1:10 in DPBS to reduce the iodine concentration rapidly, then immediately injected into a Slide-A-Lyzer Dialysis Cassette (Thermo) and dialysed three times against DPBS (>1 hr, >3 hr and then >12 hr) to remove remaining iodine. Fish were injected with 0.1 ml of the treated samples (1.0×10^7 copies per fish), and positive control was non-treated virus of the same batch, concentration and dialysis.

2.10 | Virocid

Virocid is a broad-spectrum disinfectant based on a combination of single- and double-chained quaternary ammonium compounds (24,86%), glutaraldehyde (10,73%) and isopropanol (14,63%) (CID Lines). PRV-1 in DPBS at a concentration of copies 2.0×10^9 copies/ml was diluted 1:1 in 4%, 3%, 2% or 1% of Virocid solution prepared in DPBS with yeast (Yeast Extract Granulated, Merck KGaA) as organic load (final concentration of 3 g/ml) to give a final concentration of 2%, 1,5%, 1% and 0.5%, respectively. The treated virus was incubated for 30 min at 4°C and then immediately diluted 1:10 in DPBS to rapidly reduce the Virocid concentration. The solution was injected into a Slide-A-Lyzer Dialysis Cassette (Thermo Fischer Scientific) and dialysed three times against DPBS (>1 hr, >3 hr and then >12 hr) to remove the remaining Virocid. Fish were injected with 0.1 ml of the treated samples (1.0×10^7 copies per fish), and positive control was non-treated virus of the same batch and concentration with yeast (3 g per ml) and dialysis.

3 | RESULTS

3.1 | Development of an in vivo challenge model to study PRV inactivation

In the pilot trial, the fish were challenged by three doses of virus: high (10^8 copies/fish), medium (10^6 copies/fish) and low dose (10^4 copies/fish) to optimize the challenge dose and sampling time points, and determine the target organs for PRV detection (Figure 1). In the high-dose group, PRV was first detected in spleen by RT-qPCR at 2 wpc (Ct-value 27.4 ± 3.1), and the peak viral RNA load was between 6 wpc (Ct-value 19.3 ± 3.1) and 8 wpc (Ct-value 20.0 ± 2.9) (Figure 1a). In the medium-dose group, viral RNA was also first detected in spleen at 2 wpc, but at lower loads than the high-dose group (Ct-value 36.4 ± 3.5). However, thereafter high viral loads were observed for the medium-dose group both at 4 wpc (Ct-value 17.7 ± 1.2) and at 6 and 8 wpc (Figure 1a). In the low-dose group, viral RNA was first detected in spleen at 4 wpc (Ct-value 33.6 ± 4.9), and at 6 and 8 wpc, the level was low (Figure 1a). The viral kinetics in spleen (Figure 1a) and head kidney (Figure 1b) were similar, but with a tendency of higher viral RNA loads in spleen, as observed for the low-dose group. Based on these data, the challenge dose was set to 10^7 copies/fish, that is between the high and medium dose, and samplings were set to 4, 6 and 8 wpc. Spleen was chosen as the targeted organ for PRV detection.

To assess the risk for detecting leftover PRV RNA from the inoculum, one group of fish was challenged with a high PRV dose (10^8 copies/fish) which had been inactivated by heat treatment at 85°C for 25 min, thus containing PRV RNA but no infectious viral particles. Only a few samples with high Ct-values were observed in this putatively inactivated group, including one spleen sample at 8 wpc with Ct-value 36.7 (Figure 1a) and two head kidney samples at 6 wpc with Ct-values 37.2 and 37.4 (Figure 1b). Furthermore, one spleen sample at 8 wpc with a Ct-value of 37.5 was obtained in a control

FIGURE 1 Development of the in vivo model. The pilot trial included three groups challenged with PRV by injection of high dose (red, 10^8 copies/fish), medium dose (yellow, 10^6 copies/fish) and low dose (green, 10^4 copies/fish), in addition to one group challenged with a high dose (10^8 copies/fish) of heat-inactivated PRV (black) and one group challenged by PBS (grey). PRV RNA measured by RT-qPCR in spleen (a) and head kidney (b), shown as individual and mean Ct-values ($n = 5$) from 2 to 8 weeks post-challenge (wpc). Dotted line at Ct-value 35 marks cut-off value
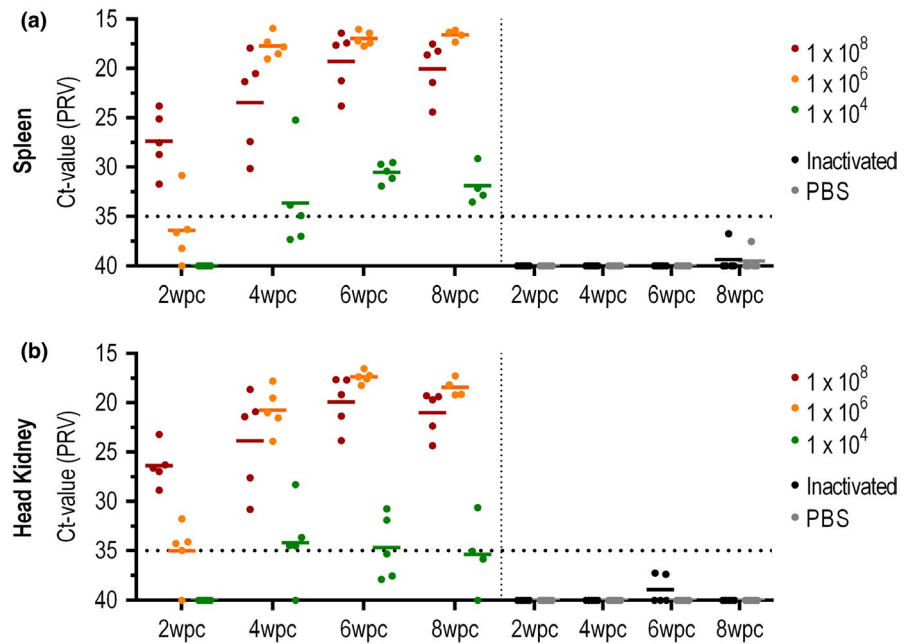
group challenged with PBS, thus devoid of PRV RNA (Figure 1a). Based on these data, a cut-off value of Ct 35 was set to mitigate the risk for false-positive samples or detection of leftover RNA from the inoculum.

3.2 | Thermal inactivation

The in vivo model was first used to study thermal inactivation of purified PRV (Figure 2a). PRV was completely inactivated after treatment at 85°C for 25 min, as viral RNA could not be detected in any samples at any time point after injection (Figure 2a). After heat treatment at 76°C for 20 min, one out of ten injected fish were PRV-positive at 4 wpc (Ct-value 32.3). However, PRV RNA was not detected at the following 6 and 8 wpc samplings (Figure 2a). After heating the virus sample at 56°C for 30 min, PRV RNA was not detected at 4 wpc, but at 6 wpc, seven out of ten fish were PRV-positive (mean Ct-value of 33.1 ± 2.2), followed by a significantly increase at 8 wpc (mean Ct-value 24.1 ± 6.7, $p < .05$) (Figure 2a). The increase in PRV RNA demonstrated replication of PRV and failure of inactivation at 56°C for 30 min.

To investigate the potential protective effect of organic content on thermal inactivation of PRV, an experiment was performed where PRV-containing blood cells were heat-treated like the purified PRV (Figure 2b). At 85°C for 25 min, one out of five fish were found positive for viral RNA at 4 wpc, but with a high Ct-value (Ct-value 34.8). There were no positive samples observed in the following samplings, which suggested complete PRV inactivation (Figure 2b). Similarly, after heating at 76°C for 20 min PRV RNA could not be detected at any samplings, also demonstrating complete PRV inactivation (Figure 2b). In conclusion, PRV in blood cells were not better protected against heat inactivation compared to purified PRV.
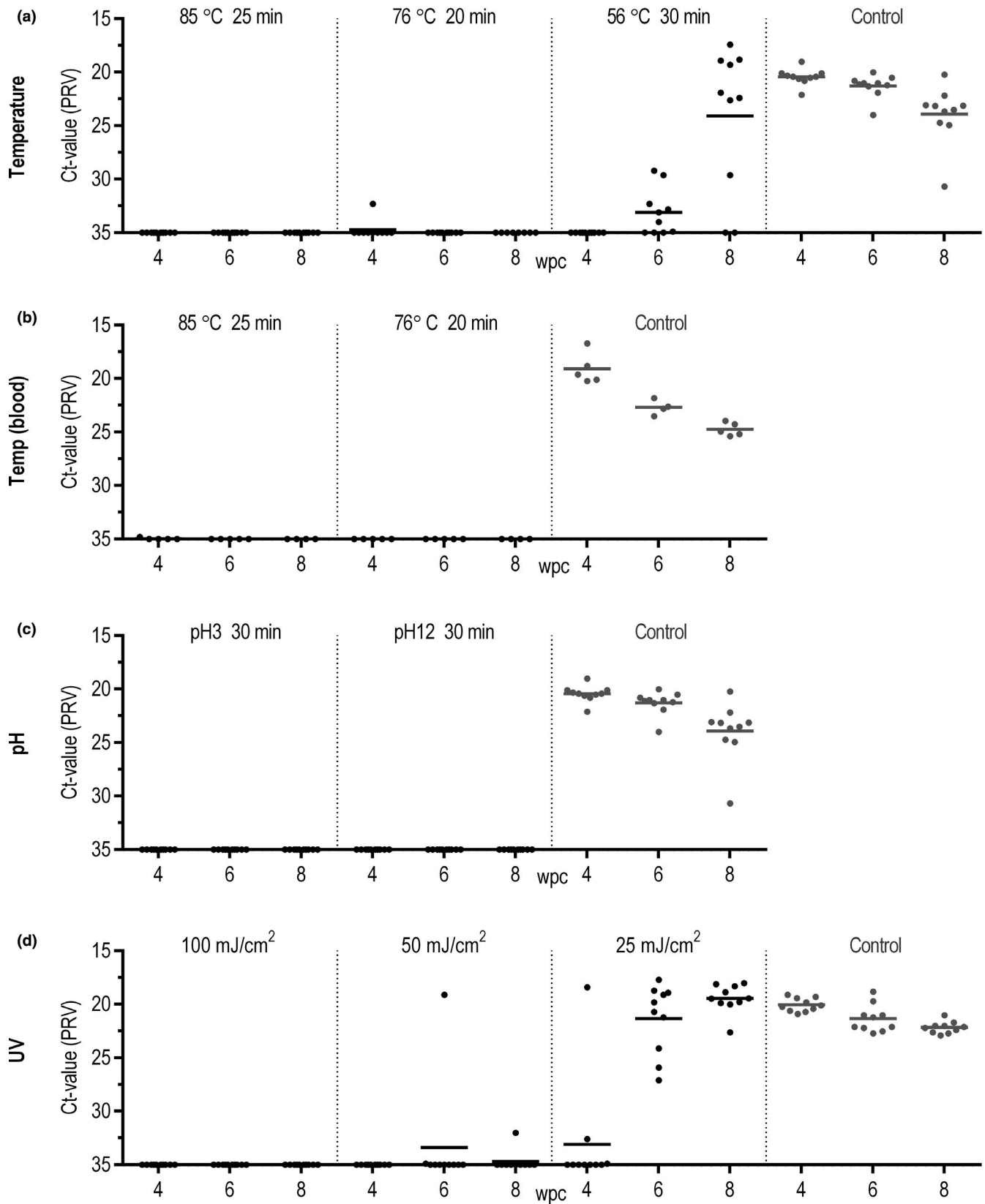
3.3 | pH inactivation

The effect of inactivation by basic and acidic conditions was tested using the in vivo model and purified PRV. Complete inactivation of PRV was observed at pH 3 for 30 min and at pH 12 for 30 min. PRV RNA was not detected at any time points after these treatments (Figure 2c).

3.4 | UV inactivation

To determine the effect of UV light on PRV inactivation, purified PRV was subjected to a range of UV doses (25 – 300 mJ/cm^2) and then tested in the in vivo model. At UV dose 300, 200 and 100 mJ/cm^2, PRV was completely inactivated with no detection of PRV RNA at any sampling (Figure 2d, only 100 mJ/cm^2 dose presented). After 50 mJ/cm^2 UV treatment, PRV RNA could not be detected at 4 wpc, but was detected in two out of ten fish at 6 wpc (Ct-values 19.1 and 34.9) and in one fish at 8 wpc (Ct-value 32.0). The detection of PRV RNA at two subsequent samplings, including one individual fish with a Ct-value of 19.1, indicated that PRV was not fully inactivated at 50 mJ/cm^2. After 25 mJ/cm^2 UV treatment, PRV RNA was detected in two out of ten fish at 4 wpc (Ct-value 18.4 and 34.9) (Figure 2d). At the two subsequent time points, all fish were positive for PRV RNA with high viral loads: 6 wpc (mean Ct-value 21.3 ± 5.2) and 8 wpc (mean Ct-value 19.4 ± 1.3). This demonstrated that the 25 mJ/cm^2 UV dose does not inactivate PRV.

3.5 | Iodine inactivation

The effect of iodine on PRV inactivation was assessed using a combination of different concentrations and exposure times. At all

FIGURE 2 Thermal, pH and UV inactivation. The in vivo model was used to test the effect of different inactivation methods. (a) Thermal inactivation of PRV at 85°C 25 min, 76°C 20 min and 56°C 30 min. (b) Thermal inactivation of blood containing PRV at 85°C for 25 min and 76°C for 20 min. (c) pH inactivation using pH 3 and pH 12 for 30 min. (d) UV inactivation using 100, 50 and 25 mJ/cm². Treated samples are shown in black and non-treated controls shown in grey (right side). PRV RNA in spleen measured by RT-qPCR shown as individual and mean Ct-values from 4, 6 and 8 weeks post-challenge (wpc) (a, c, d: *n* = 10, b: *n* = 5)

concentrations tested, including 200, 100 and 50 ppm using 10-min exposure time, PRV was completely inactivated, as shown by the absence of viral RNA (Figure 3a). Similarly, PRV was completely inactivated when exposed to 100 ppm for 20, 10 or 5 min (Figure 3a).

3.6 | Virocid disinfectant

Virocid, a commercially available disinfectant based on quaternary ammonium, glutaraldehyde and isopropanol, was tested for the effect on PRV inactivation. PRV was completely inactivated, with no detection of viral RNA at any sampling, after exposure to 2%, 1.5% and 1% Virocid for 30 min at 4°C (Figure 3b, only 1.5% and 1%

presented). When using 0.5% Virocid (30 min exposure, 4°C), PRV RNA could not be detected at 4 or 6 wpc, but all fish were positive at 8 wpc (mean Ct-value 31.5 ± 5.4). This suggests that a 0.5% Virocid treatment does not sufficiently inactivate PRV.

4 | DISCUSSION

In the present study, we developed an in vivo model to study inactivation of PRV and generated data on PRV resistance to treatment by heat, high/low pH, UV, iodine and Virocid.

The study of virus inactivation requires a model to study and quantify infectivity, which is usually a cell culture. For non-cultivable
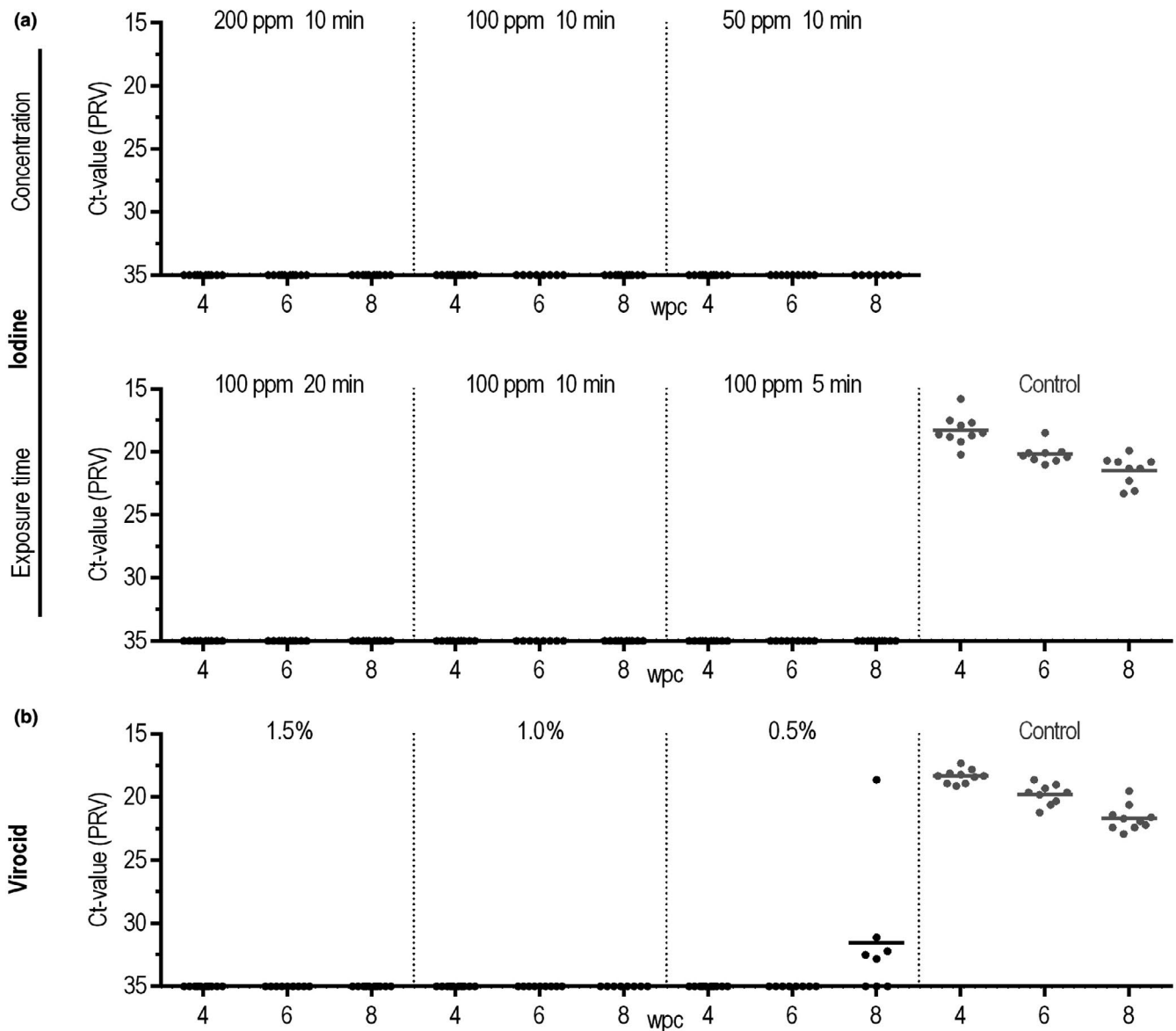
FIGURE 3 Iodine and Virocid inactivation. The in vivo model was used to test inactivation by iodine and Virocid treatment. (a) Iodine inactivation was tested at the following concentrations: 200 ppm, 100 ppm and 50 ppm with 10-min exposure (top), and the following exposure times: 100 ppm for 20 min, 10 min and 5 min (bottom). (b) Virocid inactivation was tested in a 30-min exposure using 1.5%, 1% and 0.5% solution. Treated samples are shown in black and non-treated controls shown in grey (right side). PRV RNA in spleen measured by RT-qPCR is shown as individual and mean Ct-values (n = 7–10) from 4, 6 and 8 weeks post-challenge (wpc)

viruses, this is not a possibility. The in vivo model developed in the present study circumvents this bottleneck by using the natural host Atlantic salmon, as the replicating entity to reveal the presence of infectious virus. However, inactivation of cultivable viruses is often presented as conditions giving 3-log (99.9%) reduction in the titer of infectious virus, while *in vivo* models merely state inactivation or not. Therefore, the input in the model, that is the number of infectious viruses used in the studies, is of pivotal importance. A pilot study was performed to optimize the study design, including standardized challenge dose, sampling time points and target organ. The model is based on detection of viral RNA by RT-qPCR of eventual replicating virus in the challenged fish. Although susceptible cell lines, if available, would be practical and generate more detailed results, experimental animals of the natural target species of the virus will represent a very sensitive detection system. In the model, a volume corresponding to 10^7 copies of viral genomic RNA in purified virus particles was injected into each fish after the various disinfection procedures. The output of the model was therefore specifically whether inactivation of a virus load of 10^7 copies of viral genomic RNA was achieved or not. It is important to note that although infectious virus may be completely inactivated, viral RNA may still be detected as RT-qPCR does not distinguish between viral RNA from inactivated or infectious virus. The assessment in the pilot study of possible detection of leftover RNA from the inoculum indicated that this can be controlled by setting the cut-off Ct-value so that detection of false positive is minimized. Altogether, the pilot study demonstrated that this model was suitable for studies of PRV inactivation.

Thermal inactivation of PRV was tested under selected relevant conditions. The temperature and time parameters required by the fish silage processing method (approved by the Norwegian Food Safety Authority), ≥85°C for ≥25 min at pH ≤ 4.0, were used (Nesse, Ringø, & Rosnes, 2012). Our study demonstrated that PRV was inactivated at 85°C for 25 min, at neutral pH, both for purified virus particles and for PRV in blood cells. When the temperature and treatment time were set to 76°C for 20 min, settings earlier suggested to be used for fresh (not silage) raw materials from aquaculture (Nesse et al., 2012), one fish was PRV-positive at 6 wpc using purified PRV, whereas no positives were detected using PRV in blood cells. Our results suggest that these conditions are borderline to complete inactivation of PRV. The final temperature tested was 56°C for 30 min, which is the parameters used for heat inactivation of complement in serum, at which PRV was not inactivated. This indicates that PRV, like its orthoreovirus compatriot MRV, but also like the fish virus IPNV, is relatively heat-stable (Attoui et al., 2012; Nygaard, Modahl, & Myrmel, 2012).

PRV was inactivated at both pH 3 and pH 12 for 30 min in the present study. In comparison, MRV is known to withstand rather extreme conditions including pH values between 2 and 9 (Attoui et al., 2012). A follow-up study with titration between pH 3 and 12 is needed to get detailed information about the pH range in which PRV is stable.

UV treatment of at least 50 mJ/cm² was required for PRV inactivation. This is similar to that of mammalian reovirus (MRV) which requires 45 mJ/cm² for 99.9% (3-log) inactivation (Harris, Adams, Sorensen, & Curtis, 1987). In comparison, UV doses from 118 up to 246 mJ/cm² has been reported to be required for 99.9% inactivation of IPNV (Liltved, Hektoen, & Efraimsen, 1995; Liltved et al., 2006; Oye & Rimstad, 2001). It should be noted the infectivity of IPNV and PRV is measured using different systems (cell culture model versus in vivo model) and viral quantification methods (TCID$_{50}$ versus qPCR) making a direct comparison difficult. Nevertheless, IPNV seems to have a higher resistance to UV irradiation. According to current regulations in Norway, most UV systems used in aquaculture are based on a dose of 25 mJ/cm² (calculated as minimum dose, that is UV dose in the flow path line receiving the lowest dose). The results from the present study suggest that this is not sufficient for complete PRV inactivation.

Iodine disinfection of fertilized salmonid eggs is an important barrier to salmon pathogens in salmonid aquaculture. Therefore, the effect of iodine treatment on purified PRV was tested in the present study. The standard treatment (100 ppm, 10 min) inactivated PRV; furthermore, the same effect was achieved using lower treatment time (5 min) or lower concentration (50 ppm). However, efficient disinfection of eggs most likely requires inactivation of both free PRV particles and PRV within erythrocytes, the main target cell for PRV (Wessel et al., 2015). If the broodstock is PRV-infected, blood contamination of sexual products macroscopically or microscopically during fertilization is a likely event. Halogens, like iodine, are known to react with organic compounds which can reduce its antiseptic activity (Gottardi, 2014). The potential reduced efficiency to inactivate free PRV in the presence of interfering organic substances or when PRV is shielded within erythrocytes is practically relevant questions which can be addressed in a follow-up study using the model presented in this paper.

The study included a test of different concentrations of Virocid and confirmed that at least 1% concentration for 30 min is needed for complete PRV inactivation under the test conditions, which included yeast as organic load. This demonstrates the applicability of the model to investigate the effect of commercially available products on PRV inactivation. It should be noted that at treatment with a lower, less efficient dose, PRV could not be detected at 4 or 6 wpc and was first detected at the final time point of 8 wpc. This raises the question of whether the duration of the trial should be extended to allow longer time to detect possible PRV replication. On a more general note, this indicates that a PRV infection can go undetected for a surprisingly long time period. A similar observation was made when one of the fish groups injected where thermal-inactivated PRV was investigated (Figure 2a; Temperature 56°C 30 min). This group was found to be PRV-negative until 6 wpc, but PRV-positive thereafter. This depicts a general limit in sensitivity of the qPCR assay and demonstrates the difficulty to document that a fish population is PRV-negative.

In conclusion, the present study provides novel information about the conditions required for PRV inactivation. This information can be useful to avoid and eradicate PRV contamination in closed aquaculture facilities and thereby limit the risk of HSMI outbreaks.

The model provides a potential framework for future inactivation studies of other non-cultivable viruses in Atlantic salmon.

CONFLICT OF INTEREST

VESO hold the aquatic research facilities in which all trials were performed. VESO is also the distributor of Virocid, one of the disinfection products tested in the study. This did not have any influence on the set-up or results of the trial. Xylem contributed with equipment and expertise to set-up of controlled UV treatment in the experiment. Xylem offers UV treatment products to the aquaculture industry. This did not have any influence on the set-up or results of the trial.

DATA AVAILABILITY STATEMENT

All data from this study are included in a supplementary file.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Wessel Ø, Hansen EF, Løvoll M, et al. Inactivation of *Piscine orthoreovirus*. *J Fish Dis*. 2020;43:1039–1048. https://doi.org/10.1111/jfd.13214