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Release and survival of infectious salmon anaemia (ISA) virus during decomposition of Atlantic salmon (*Salmo salar* L.)

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ABSTRACT

Infectious salmon anaemia (ISA) is a serious viral disease of farmed Atlantic salmon, *Salmo salar* L., caused by infectious salmon anaemia virus (*Isavirus*, Orthomyxoviridae). An ISA outbreak may vary when it comes to clinical manifestations and mortality levels. Some outbreaks have an aggressive development with an escalating mortality level (up to 90%). At these sites, efficient removal of dead fish can be a major challenge. Removal of dead fish is an important fish health preventive measure implemented at marine sites. In the present study we have examined the infection risk the dead fish may represent for the remaining fish at the site by examining both tissues from fish that died from ISA, and from the surrounding water (0–120 h *post mortem*). The aim was to examine for how long infectious virus particles could be retrieved from dead salmon as this could indicate if such material may pose a risk for spreading of ISA virus. Although viral RNA was detectable in water from decomposing salmon by real-time RT-PCR, no infectious particles could be obtained from water either by inoculating cell cultures or by injecting water samples into fish. Nevertheless, the study showed that infectious virus particles could be retrieved from heart tissue of dead fish for more than 4–5 days *post mortem*.
Thus, daily removal of dead fish is an important preventative measure during ISA outbreaks. It is also of importance to remove moribund fish with ISA as these continue to produce and shed virus and may represent a greater risk with respect to the transmission of virus than the fish that are already dead.

1. Introduction

Infectious salmon anaemia (ISA) is a disease characterised by severe anaemia of farmed Atlantic salmon (*Salmo salar* L.) and which may lead to high mortality in an affected population. The etiological agent is the ISA virus, an enveloped, negative single stranded segmented RNA virus in the genus *Isavirus*, family Orthomyxoviridae (King et al., 2012; Krossoy et al., 1999; Mjaaland et al., 1997). ISA is a challenge in all main farming areas around the North Atlantic and in Chile (Bouchard et al., 2001; Godoy et al., 2008; Kibenge et al., 2001; Lovely et al., 1999; Lyngøy, 2002; Mullins et al., 1998; Ritchie et al., 2001; Rodger et al., 1998; Rowley et al., 1999; Thorud and Djupvik, 1988). Outbreaks of ISA predominantly affect the marine phase of production when the salmon is most valuable for the farmers (growth phase). The main reservoir for ISA virus in farming seems to be farmed Atlantic salmon (Plarre et al., 2005), and there is evidence that the virus is trans-generationally (vertically) transmitted (Nylund et al., 2006; Plarre et al., 2012; Søfteland, 2005; Vike et al., 2009) in addition to being horizontally transmitted through water or by vectors/fomites (Aldrin et al., 2010, 2011; Gustafson et al., 2005, 2007; Jarp and Karlsen, 1997; Lyngstad et al., 2008, 2011; Mardones et al., 2009, 2011; McClure et al., 2005; Murray et al., 2010; Nylund et al., 1993; Scheel et al., 2007; Vagsholm et al., 1994).

An ISA outbreak may vary when it comes to clinical manifestations and mortality levels. Some outbreaks have an aggressive development with escalating mortalities up to 90% (EFSA, 2012; Rimstad and Mjaaland, 2002; Vagsholm et al., 1994, pers. obs.), where all cages may be affected simultaneously resulting in a total collapse of production at the entire site. At these sites, efficient removal of dead fish can be a major challenge. Moderate mortality is also often seen where only single cages may be affected at a site (Lyngøy, 2002, pers. obs). Experience from Norway shows that ISA virus may spread slowly within a sea site, and a neighbouring cage may not be infected for up to 30 days after manifestation in an adjacent cage (Thorud and Håstein, 2003, pers. obs.). The mortality development seems to be temperature dependent, and most ISA outbreaks seem to occur when the water temperature is between 10 and 15 °C (optimal temperature for replication of ISA virus) (Falk et al., 1997; Thorud, 1991). However, ISA outbreaks have been reported to occur at temperatures as low as 6 °C (pers. obs.). Under laboratory conditions it has been shown that replication of ISA virus was reduced significantly at 20 °C (Falk et al., 1997). The same pattern may be seen

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from farming sites where ISA mortality can decrease and increase when the temperature rises above or drops below 15 °C respectively (Poppe et al., 1999).

It is well documented that Atlantic salmon suffering from ISA shed virus into their environment and subsequently infect other naïve salmon (Gregory et al., 2009; Jones and Groman, 2001; Nylund et al., 1994; Thorud and Djupvik, 1988; Thorud and Håstein, 2003). Salmon suffering from ISA may start to shed virus as early as seven days postchallenge (Gregory et al., 2009). To learn more about ISA virus transmission this study examines tissues from ISA diseased Atlantic salmon during decomposition, and focuses on the possible release of ISA virus particles into the water (0–120 h *post mortem*). The aim was to examine for how long infectious virus particles could be retrieved from dead salmon and the surrounding water as this could indicate if such material may pose a risk for the spreading of ISA virus at aquaculture sites.

2. Materials and methods

2.1. Virus

The infectious salmon anaemia virus isolate used in this study was a Chilean field isolate CH0108 (accession # EU851083). The virus was obtained from Atlantic salmon tissue sent from Chile to Norway on dry ice, isolated in ASK-cells and stored in liquid nitrogen. This ISA virus isolate has been described previously (Lauscher et al., 2011; Vike et al., 2009; Wolf et al., 2013). Fish were intraperitoneally (i.p.) infected with 0.1 mL of 3×10^6 virus per mL measured using $TCID_{50}$. Ten of these ISA positive fish were used further in this study *post mortem*.

2.2. Fish and experimental set-up

The Atlantic salmon were supplied by a local producer, and had an average weight of approximately 50 g. The fish were, prior to the experiment, screened by real-time RT-PCR and found negative for infectious pancreas necrosis virus and salmonid alphavirus. The fish were kept at the Industrial- and Aquatic Research Laboratory (ILAB) in Bergen, Norway. The animal experiment had been approved by the Norwegian Animal Research Authorities (NARA) (ID 5114). Fish were infected after 6 weeks of acclimatisation at 12 °C in freshwater. Ten fish were included in the experiment. Each fish was placed individually in an autoclaved glass container filled with 5 L of freshwater, without aeration. The glass containers with static, non-aerated water were kept at two different temperatures (7 and 18 °C), with five replicas for each temperature. For convenience, these individuals will be referred to as F1 to F10 throughout the manuscript. F1–F5 fish were kept at 7 °C and F6–F10 at 18 °C. Two fish kept at 7 °C had died from ISA (F1 and F2) 24 days postinfection (d.p.i.) whereas the remaining fish were moribund with ISA, and were killed by a blow to the head at 25 d.p.i. The fish were left to decompose under these conditions for up to 120 h. F1 and F2 were kept for 120 h, while F3–F10 fish were kept for 96 h.

2.3. Sampling of tissues and water from decomposed fish

Water samples (triplicates of 50 mL) were carefully collected with a sterile glass pipette from the glass containers with decomposing fish at various time points *post mortem*: 0, 1, 3, 6, 24, 48, 96/120 h — hereafter abbreviated as T0, T1, T3, T6, T24, T48 and T96/T120. One sub-sample was processed for real-time RT-PCR according to the method described in Andersen et al. (2010), using electropositive 1 MSD filters followed by downstream real-time RT-PCR. The second 50 mL aliquot was used to test for virus infectivity by inoculation onto cell culture and to challenge naïve fish. The third 50 mL aliquot was kept at −80 °C for long-term storage.

Tissues were sampled *post mortem* (before decomposition at T0 and at T96/T120). Gills were sampled from all fish initially (T0), but due to the high degree of decomposition of the fish at the end of the experiment, further gill samples could not be retrieved. Thus, heart samples were collected instead of gills at the end of the experiment (T96/120).

Prior to the water filtration, a test was performed to ensure that cell culture medium (EMEM) containing 2% FBS was able to reproducibly release virus particles from the electropositive 1 MDS filters. Water samples were spiked with ISA virus with a $TCID_{50}$ concentration in a range of 5.3×10^4 virus mL^{-1} to 6.6×10^6 virus mL^{-1} and the virus recovery after filtration was on average 36.5%. The reading of the $TCID_{50}$ dilution series was done by using IFAT (Falk et al., 1998).

2.4. Virus infectivity of water samples during the decomposition process

2.4.1. Water samples inoculated onto cell culture

The ASK-cells were cultured with Eagle's minimum essential medium (EMEM) from Sigma, supplemented with HEPES buffer (10 mM), non-essential amino acids (0.1 mM/100 μM), L-glutamine (4 mM) and gentamicin (50 μg/mL) with 10% FBS in 75 cm^2 tissue culture flasks (Nunc) at 15 °C. Filtered water samples eluted in EMEM with 2% FBS from all samplings times (T0, T1, T3, T6, T24, T48 and T96/T120) were inoculated onto ASK-cells (0% FBS) to examine if infective ISA virions were present (Devold et al., 2000).

Twenty-four well cell culture plates (Nunc) with ASK-cells were inoculated with 600 μL water sample per well, in triplicate. Positive and negative controls were also included. The plates were incubated at 15 °C. A 100 μL supernatant was sampled for real-time RT-PCR analysis after passage 1.

2.4.2. Fish challenged with water samples or heart homogenate

In the experimental set-up, 230 Atlantic salmon were divided into 8 tanks (Table 1). The fish were unvaccinated smolts (50 g) adapted to sea water (30‰ salinity) at 10 °C. They were acclimatised in 0.15 m^3 tanks with a flow rate of 0.8 L^{-1} kg^{-1} fish min^{-1} for one week prior to the startup of the experiment. Prior to i.p. injection all fish were anaesthetised with Metacaine MS-222. The animal experiments had been approved by NARA (ID 2830).

The filtered water samples eluted in EMEM with 2% FBS used in the *in vitro* study of ISA virus infectivity in ASK-cells were also used in a fish

Table 1
Overview of the experimental set-up for Atlantic salmon challenged with concentrated water samples from all time points (T1–T96/120) from fish F1, F2, F3, F4 (incubation temperature 7 °C) and F8 (incubation temperature 18 °C). A total of 230 fish were included in the experiment and these were divided into 8 tanks. Thirty different inocula from different water samples were injected into fish (five fish per inoculum). Eighty of the fish were naïve cohabitating control fish, 10 per tank, injected with EMEM.

Tank	Inocula	N	Total N	Tank	Inocula	N	Total N
1	T1 F1	5	30	5	T6 F3	5	30
	T1 F2	5			T6 F4	5	
	T3 F1	5			T24 F3	5	
	T3 F2	5			T24 F4	5	
	EMEM	10			EMEM	10	
2	T6 F1	5	30	6	T48 F3	5	30
	T6 F2	5			T48 F4	5	
	T24 F1	5			T96 F3	5	
	T24 F2	5			T96 F4	5	
	EMEM	10			EMEM	10	
3	T48 F1	5	30	7	T1 F8	5	25
	T48 F2	5			T3 F8	5	
	T120 F1	5			T6 F8	5	
	T120 F2	5			EMEM	10	
	EMEM	10					
4	T1 F3	5	30	8	T24 F8	5	25
	T3 F3	5			T48 F8	5	
	T1 F4	5			T96 F8	5	
	T3 F4	5			EMEM	10	
	EMEM	10					