Heart and skeletal muscle inflammation in Atlantic salmon, *Salmo salar* L.: a new infectious disease

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Abstract

Heart and skeletal muscle inflammation (HSMI) is a disease syndrome of unknown aetiology first observed in farmed Atlantic salmon, *Salmo salar*, in 1999. In the present study we have demonstrated for the first time that HSMI is an infectious disease. It was induced in Atlantic salmon post-smolts after injection with tissue homogenate from farmed Atlantic salmon previously diagnosed with HSMI. The lesions were also induced in cohabitating salmon given a corresponding injection without tissue homogenate. Six weeks post-challenge the fish that had been injected with tissue homogenate developed a serious epicarditis and myocarditis with mononuclear cell infiltrations in compact and spongy layers of the heart. Similar lesions were found in cohabitants after 10 weeks. The lesions were consistent with samples from field outbreaks of HSMI. No lesions were found in control fish. A viral aetiology is strongly suggested, as no difference in disease induction between an inoculum containing antibiotics and a non-treated inoculum was found. Further investigations are required in order to make conclusions regarding the cause and pathogenesis of HSMI.

Keywords: Atlantic salmon, heart and skeletal muscle inflammation, pathology, transmission.

Introduction

During 1999, outbreaks of a previously undescribed disease in sea caged Atlantic salmon, *Salmo salar* L., were reported along the Norwegian West coast. The disease was given the name ‘heart and skeletal muscle inflammation (HSMI)’ because of the characteristic histopathological lesions that occurred in affected fish. The number of reported outbreaks is increasing [as recorded by the National Veterinary Institute in Norway (NVI), 2003]. In 2002, fish from 41 salmon farms along the Norwegian coast were diagnosed with HSMI at the NVI laboratories. Disease occurrence has been reported the whole year round, but seems to be most common during spring and early summer, 5–9 months after sea transfer. Morbidity is high, as most fish in affected sea cages show histopathological lesions in heart and skeletal muscle. Mortality varies from almost insignificant up to 20%. Management stress seems to increase the mortality rate and prolong the healing process during outbreaks.

Macroscopic changes include a pale heart with loose texture, pericardial haemorrhage, ascites and a pale or stained liver. Haematocrit levels are usually normal.

The most significant histopathological lesions of HSMI are found in heart and red skeletal muscle. In the heart, necrosis of muscle fibres and a massive inflammatory response involves both compact and spongy layers of the ventricle. Infiltrations are mainly mononuclear. An extensive epicarditis is usually found in association with the myocarditis. Red muscle inflammation follows the same pattern as seen in the heart, but is not a consistent finding. Other lesions include focal liver necrosis and
circulatory disturbances such as oedema and erythrocyte accumulation in several organs (Kongtorp, Taksdal & Lyngøy 2004). Investigations regarding the cause of HSMI have previously only been speculative. Field observations indicate that the disease has a contagious nature (Kongtorp, Taksdal & Lyngøy 2004). The pathological changes resemble those found in pancreas disease (PD) (Ferguson, Roberts, Richards, Collins & Rice 1986; Rodger, Murphy, Drinan & Rice 1991; Murphy, Rodger, Drinan, Gannon, Kruse & Korting 1992; Rodger, Turnbull & Richards 1994; McLoughlin, Nelson, McCormick, Rowley & Bryson 2002) and cardiomyopathy syndrome (CMS) (Ferguson, Poppe & Speare 1990), but do not share all features of the two diseases (Kongtorp et al. 2004). Results from a pilot study conducted in 2001 (A. Kjerstad, A. Guttvik, H. Skjelstad, T. Taksdal & K. Falk, unpublished results), indicate that HSMI is infectious.

This paper describes a challenge study conducted in 2002. In an experimental challenge, we demonstrated that HSMI is transmissible both by injection and cohabitation.

**Materials and methods**

**Preparation of tissue homogenate**

Organ homogenates were made from Atlantic salmon displaying lesions consistent with HSMI (Fig. 1A, E) from a field outbreak. Atrial and ventricular myocardium from seven fish (50% wv) were homogenized in Leibovitz L-15 cell culture medium (L-15), and centrifuged at 2500 g for 7 min. The resulting supernatant was further diluted in L-15 media with and without gentamicin (final concentration 50 µg mL⁻¹). L-15 media with and without gentamicin in the same concentration was used as control inocula.

**Transmission trial**

Experimental fish originated from a fish farm from which no signs of disease had been reported. They had hatched in January 2001 and were transported to the research station VESO Vikan (Namsos, Norway) in mid February 2002. The fish were transferred to sea water in late April 2002. On commencement of the challenge study in late June 2002, they had an average weight of 90 g. A total of 500 salmon were placed in three tanks, measuring 1 x 1 x 0.5 m. Water flow through the tanks was 0.8 L kg fish⁻¹ min⁻¹. The temperature ranged from 10 to 12 °C during the study and the salinity in the tanks was 33%o on average. The inoculate (0.2 mL) was injected intraperitoneally.

In tank 1, experimental fish were injected with inocula supplemented with gentamicin. One hundred fish were injected with tissue homogenate. An additional 100 fish were injected with a control inoculum and placed in the tank as cohabitants. Similarly, 200 fish were injected with antibiotic-free inocula in tank 2. One hundred fish were challenged with tissue homogenate and 100 fish with L-15 media. A control group of 100 fish was given a corresponding injection with L-15 media and kept in a separate tank. After inoculation of the fish, data relating to mortality, feeding, water temperature and salinity were recorded daily.

**Blood and tissue sampling**

Samples were taken 1, 2, 4, 6, 8 and 10 weeks post-challenge. At each sampling, five fish from each of the five groups were killed by a blow to the head and bleeding. Blood samples were collected from the caudal vein for haematocrit measurements. Tissue samples from heart, skeletal muscle, mid kidney and pyloric caeca with pancreas were collected and fixed in 10% neutral phosphate buffered formalin. Fixed samples were prepared for histological examination by standard paraffin wax techniques and stained with haematoxylin and eosin. Sampled fish were classified histologically as diseased or non-diseased based on the presence of myocarditis with mononuclear cell infiltration and myocardial necrosis consistent with HSMI (Kongtorp et al. 2004). The presence of an associated serious epicarditis was used to confirm the diagnosis.

One, 2 and 4 weeks after challenge, samples from mid kidney were cultivated on blood agar plates with 2% NaCl at 10 °C for 4–6 days. In the event of mortalities during the study, the same procedure was performed on the dead fish. Tissue from mid kidney was used to inoculate cell cultures. Samples from 10 fish that had been challenged by inoculation and sampled 4 and 8 weeks post-infection (p.i.) were diluted in L-15 media, homogenized and centrifuged at 1000 g for 10 min. As infectious pancreatic necrosis virus (IPNV) is ubiquitous in Norwegian salmon farms (Melby, Krogstad, Hästén & Stenwig 1991), rabbit antisera against the WB, Ja and N1 serotypes
Figure 1 *Salmo salar*. Micrographs of sections from experimental fish (haematoxylin and eosin). (A–D) Heart. (A) Compact myocardium from fish used as inoculate material. There is a severe diffuse myocarditis and epicarditis. (B) Compact myocardium and epicardium of control fish 10 weeks after study commencement. (C) Compact myocardium of the ventricle in cohabitant fish 10 weeks after study commencement. Myocardial fibres are degenerative and there is a massive inflammation in the surrounding tissue. (D) Ventricle of a cohabitant fish 10 weeks after challenge. There is a severe epicarditis and a diffuse hypercellularity in the underlying compact myocardium. (E–H) Red skeletal muscle. (E) Red skeletal muscle from fish used as inoculate material. There is extensive degeneration and cellular infiltration. (F) Red skeletal muscle in control fish 10 weeks after challenge. (G) Red skeletal muscle in fish injected with tissue homogenate from diseased fish 12 weeks earlier. Focal inflammation and myocyte degeneration. (H) Red skeletal muscle in cohabitant fish 10 weeks after challenge. A degenerative myocyte shows evidence of nuclear lysis. There is an infiltration of mononuclear cells in the myocyte fibre and surrounding interstitium. a, epicardium; b, compact myocardium; c, myocyte necrosis.
of IPNV [mAb N1-H8 (Christie, Ness & Djupvik 1990), kindly provided by K.E. Christie, Intervet Norbio AS] were added to the tissue suspension in appropriate dilutions to inhibit replication of this virus in the cell cultures. The inoculate was used to infect the following cell cultures: rainbow trout gill (RT-Gill), chinook salmon embryo (CHSE-214), Atlantic salmon head kidney (ASK-2) (Devold, Krossøy, Aspehaug & Nylund 2000), epithelioma papulosum cyprini and fat head minnow. The cells had been grown at 20 °C in L-15 supplemented with 10% foetal calf serum, L-glutamine (4 mm) and gentamycin (50 μg mL⁻¹). After inoculation with tissue homogenates, cells were incubated at 15 °C. All cultures were blind passaged twice at 14 day intervals.

Results

The challenged fish collected sequentially by random sampling developed lesions consistent with HSMI (Table 1). The lesions appeared 6 weeks p.i. in fish injected with diseased material and 10 weeks p.i. in cohabitant groups. A severe epicarditis could be seen in all fish that had developed myocardial lesions. Mild to moderate epicarditis was present in fish showing signs of early myocardial damage. Fish with no myocardial lesions did not have epicarditis. No lesions were detected in fish from the control tank (Fig. 1B, F).

In fish injected with tissue homogenate, the heart lesions detected at 6 weeks p.i. were mild to moderate. Only two fish had lesions that could clearly be distinguished as HSMI, but all inoculated fish in the sample showed signs of early myocardial damage. Fish with no myocardial lesions did not have epicarditis. No lesions were detected in fish from the control tank (Fig. 1B, F).

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Investigations of the fish that had died during the study showed mainly a mixed flora of bacteria, including Moritella viscosa (Table 2). The cell culture trial gave no conclusive results. The

<table>
<thead>
<tr>
<th>Weeks after challenge</th>
<th>Tank 1, inoculum treated with gentamycin at challenge</th>
<th>Tank 2, inoculum not treated at challenge</th>
<th>Tank 3, inoculum not treated at challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Injected with tissue homogenate</td>
<td>Injected with tissue homogenate</td>
<td>Injected with tissue homogenate</td>
</tr>
<tr>
<td>1 week</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2 weeks</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4 weeks</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6 weeks</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>8 weeks</td>
<td>4</td>
<td>3</td>
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</tr>
<tr>
<td>10 weeks</td>
<td>4</td>
<td>5</td>
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</tr>
<tr>
<td>12 weeks</td>
<td>5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>In total</td>
<td>14</td>
<td>9</td>
<td>14</td>
</tr>
</tbody>
</table>

Table 1 Challenge study of heart and skeletal muscle inflammation (HSMI). Number of salmon showing histopathologic changes in the heart consistent with HSMI at each sample date. The sample size was five fish in each group at each sampling.
haematocrit values of the experimental fish did not differ significantly from the control fish.

**Discussion**

In this study we have demonstrated the transmissibility of a newly discovered disease in farmed Atlantic salmon recognized by extensive inflammation in the heart and skeletal muscle. We have shown that HSMI can be induced in naïve Atlantic salmon by intraperitoneal injection of homogenized material from diseased fish and by cohabitation. The verification of transmission was based on histopathological findings of heart lesions that were consistent with field cases. The control group had been given similar treatment and environmental conditions as the experimental groups, but did not show any signs of disease during the study. From this we conclude that HSMI is an infectious disease.

The first heart lesions appeared at 6 weeks p.i. in the group that had been inoculated with tissue homogenate from diseased fish. Corresponding lesions were found in the cohabitants at 10 weeks p.i., showing that HSMI can be transmitted via water. Treatment of the inoculum with a broad-spectrum antibiotic prior to inoculation did not affect the transmission of HSMI from sick to healthy fish, as there was no significant difference in the appearance of lesions between the two challenged groups. Bacteriological investigations were negative in all the sequentially sampled fish. This is consistent with investigations from field outbreaks of HSMI, from which no specific bacteria have been isolated (personal observations). These results strongly suggest a viral aetiology of HSMI. Inoculation of cell cultures in the present study did not reveal any causal agent, but were conducted on a small scale. Further, the time delay in the disease outbreak of the cohabitating fish compared with the fish that had been injected with tissue homogenate indicates a peak in possible virus shedding occurring at 4 weeks p.i. From studies of infectious salmon anaemia it has been observed that viraemia occurs concurrently with a peak in virus shedding (Dannevig, Falk & Skjerve 1994; Rimstad, Falk, Mikalsen & Teig 1999; Devold et al. 2000; Raynard, Snow & Bruno 2001). The conclusion

**Table 2** Challenge study of heart and skeletal muscle inflammation. Bacterial growth on blood agar from fish that died during the study

<table>
<thead>
<tr>
<th>Isolated bacteria</th>
<th>Tank 1, inoculum treated with gentamycin at challenge</th>
<th>Tank 2, inoculum not treated at challenge</th>
<th>Tank 3, inoculum not treated at challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Injected with tissue homogenate</td>
<td>Cohabitants</td>
<td>Injected with tissue homogenate</td>
</tr>
<tr>
<td>Moritella viscosa</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mixed flora</td>
<td>4</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Moritella viscosa</td>
<td>5</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>No bacterial growth</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

**Figure 2** Challenge study of heart and skeletal muscle inflammation. Cumulative mortality in challenged groups. In tank 1, the inocula had been treated with gentamycin prior to injection into the experimental fish. In tank 2, the inocula were injected without gentamycin additives. ■ Inoculated with tissue homogenate, □ cohabitants.
that HSMI is transmissible under experimental conditions without causing mortality is supported by the results from the pilot study conducted in 2001 (A. Kjerstad, A. Guttvik, H. Skjelstad, T. Taksdal & K. Falk, unpublished results). In this study, the heart lesions were transmitted both by injection of homogenized tissue from diseased fish and by cohabitation.

In the present study, mortalities were observed among the challenged fish. The cause of death was, however, not likely to be HSMI, as the morbidity because of HSMI was equal in the two challenged groups. After 2 weeks p.i., no fish died in tank 1, while the number of deaths in tank 2 was high throughout the study (Fig. 2). Overall, the number of deaths was considerably higher in the group of fish that had been injected with an inoculum without antibiotics, compared with the antibiotics group. Moritella viscosa, a Gram-negative bacterium that has been associated with winter ulcer in Atlantic salmon (Benediktsdóttir, Verdonck, Spröer, Helgason & Swings 2000; Lunder, Sørum, Holstad, Steigerwalt, Mowinckel & Brenner 2000), was isolated from several of the dead fish, but not from any of the sequentially sampled and killed fish in the study. Winter ulcer most often occurs during the winter months, when the sea temperature is low (Salte, Rørvik, Reed & Norberg 1994; Bruno & Poppe 1996). In a challenge study conducted on winter ulcer in Norway, M. viscosa was shown to infect healthy fish at a water temperature of 10 °C (Lunder, Evensen, Holstad & Håstein 1995).

Under experimental conditions, M. viscosa is most readily isolated from fish that have been kept at low water temperatures (A. Guttvik, unpublished results). The present study of HSMI was conducted at water temperatures ranging from 10 to 12 °C. Taking these findings together, there is likely to be an association between the mortality and the isolation of M. viscosa.

In the present study, the HSMI diagnosis was based on the findings of heart lesions, because of the non-consistency of muscular lesions in field outbreaks. The lesions were milder than those reported from field cases, but were consistent with naturally occurring HSMI, presenting focal or diffuse cellular infiltration in all layers, accompanied by myocyte necrosis. In the skeletal muscle, there were only mild to moderate lesions, never reaching the severity that can be seen in field cases. Similar experiences have been made from challenge studies of PD (McLoughlin, Nelson, McCormick & Rowley 1995; Desvignes, Quentel, Lamour & Le Ven 2002). This may indicate that lesions in the skeletal muscle appear at a later stage in the pathogenesis, or that agent concentration in a field outbreak could be higher than that of this experiment, causing a potentially more aggressive disease. It also underlines the fact that a HSMI diagnosis must not be based solely upon lesions in the skeletal muscle.

The morbidity of HSMI seems to be high. In field outbreaks, it has been observed that most fish in an affected sea cage have lesions in the heart and skeletal muscle. Field observations have also shown that surviving fish in affected sea cages will recover (T. Taksdal, unpublished data). Histopathological lesions have been found in fish for a long time after symptomatic recovery. Examination of the samples from the challenge study supports the field observations of high morbidity. During the last weeks of the study, diseased fish were frequently detected in the samples by random sampling of fish from the experimental tanks.

Pancreas disease and CMS are the most relevant differential diagnoses to HSMI at the histopathological level. Cardiac lesions associated with PD (Rodger et al. 1991; Murphy et al. 1992; McLoughlin, Nelson, Rowley, Cox & Grant 1996; McLoughlin et al. 2002) show great similarity to the lesions observed in HSMI outbreaks. Pancreatic lesions are, however, important hallmarks of PD, as indicated by the name (Munro, Ellis, McVicar, Mc Lay & Needham 1984). Also, experimental trials on PD have consistently resulted in pancreatic lesions (Boucher, Raynard, Houghton & Baudin Laurencin 1995; Murphy, Drinan & Gannon 1995; McLoughlin et al. 1996; Desvignes et al. 2002) As pancreas was normal in all samples in the present study of HSMI, the hypothesis of a possible connection between the two diseases is not supported. This result is consistent with investigations from field outbreaks (Kongtorp et al. 2004). To further distinguish between HSMI and PD, RNA was extracted from frozen kidney samples of 15 fish that had been sampled 4 and 8 weeks after challenge and tested by reverse transcriptase polymerase chain reaction for salmon pancreas disease virus (SPDV) according to standard procedures at the NVI. SPDV was not detected in any of the samples.

When HSMI was first recognized as a unique disease in 1999, only a few locations in a limited area of the Norwegian coast seemed to be affected. Since then, HSMI has been diagnosed at an
increasing number of locations and in a large geographical area, indicating an infectious nature. In 2002, most parts of the coastline had outbreaks of disease that were verified as HSMI. The disease seems to be having an increasing impact on the health status of Norwegian salmon farms and will consequently be of economic significance for the industry. Some reports indicate that HSMI has a tendency to reappear annually at the same locations (A. Kjerstad, unpublished data). It is not known, however, whether this is because of a carrier status in some fish, persistence in the farm cages, or if it represents a reintroduction of the infection. The present study confirms the infectious nature indicated by the field observations. HSMI is therefore a potential threat to salmon farms both within and outside Norway, following water-born spread of the causal agent. Further investigations are required in order to make conclusions regarding the aetiology and pathogenesis of HSMI.

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References


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