Atlantic salmon paramyxovirus (ASPV) infection contributes to proliferative gill inflammation (PGI) in seawater-reared *Salmo salar*

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ABSTRACT: Proliferative gill inflammation (PGI) causes significant losses in farmed Atlantic salmon *Salmo salar* L. in Norway, especially during the first months following seawater transfer. The aetiology is apparently multifactorial, including infection with chlamydia-like bacteria and Atlantic salmon paramyxovirus (ASPV). In the present study, gills from diseased fish from 3 farms on the western coast of Norway were sampled. The pathological changes were briefly described and the aetiologic significance of ASPV studied by immunofluorescent staining of cryosections and by immunohistochemistry on sections of formalin-fixed and paraffin-embedded tissue. The pathological changes were macroscopically characterized by palour of the gills, and histologically by inflammation, circulatory disturbances, cell death and epithelial cell proliferation. ASPV was demonstrated in fish from all farms studied, as immunostaining consistent with ASPV was obtained in lamellar epithelial and endothelial cells of pathologically altered tissues. It is concluded that ASPV is at least a contributing cause of PGI. As far as we know, this is the first demonstration of fish disease related to infection with a paramyxovirus.

KEY WORDS: Atlantic salmon · *Salmo salar* · Proliferative gill inflammation · PGI · Epitheliocystis · Atlantic salmon paramyxovirus · ASPV · Immunofluorescence · Immunohistochemistry

INTRODUCTION

Proliferative gill inflammation (PGI) denotes a disease of seawater-reared Atlantic salmon *Salmo salar* L. in Norway. PGI is not a notifiable disease and estimates of losses for the entire industry are not available. According to diagnostic investigations by local fish health services and the National Veterinary Institute, the disease has been present since the 1980s and occurs in all coastal regions where salmon farming is carried out. It occurs especially during the autumn and is an important cause of loss related to mortality and reduced growth rate in fish transferred to seawater the previous spring. Outbreaks may last for months (Nygaard & Flesjå 1997, Nygaard 2000). Extrapolations from diagnostic investigations in the county of Møre og Romsdal in 2003 indicate that approximately 150 farms in Norway may have been affected by PGI that year (Kvellestad et al. 2004).

Histological examinations of gills indicate a multifactorial aetiology of PGI. Epithelial cell inclusions (i.e. epitheliocysts) with chlamydia-like bacteria (Hoffman et al. 1969, Nylund et al. 1998, Draghi et al. 2004) are frequently but inconsistently seen in large numbers, suggesting that this agent is at least associated with the disease. Thus, this disease has often been termed ‘epitheliocystis’. Although protozoan ectoparasites such as trichodinids and *ichthyobodo necator*-like...
parasites may also be inconsistently observed at gill surfaces (Kvellestad et al. 2004), their role as primary aetiopathological factors is doubtful. Recently, a previously unknown virus, Atlantic salmon paramyxovirus (ASPV), was isolated from the gills of fish with PGI (Kvellestad et al. 2003), suggesting a possible viral involvement in development of this disease. However, due to the fact that the above-mentioned agents are inconsistently detected and their aetiopathological significance has not been fully investigated, we currently use the descriptive term ‘proliferative gill inflammation’ for this disease in order to reflect the unsolved aetiology.

This paper briefly describes the gill pathology of PGI as it occurred in fish selected from 3 seawater farms. A rabbit hyperimmune serum against ASPV was produced, characterized and used to detect viral antigen in gills by an indirect immunofluorescence (IIF) test on cryosections and immunohistochemistry (IHC) on gills by an indirect immunofluorescence (IIF) test on sections of formalin-fixed and paraffin-embedded tissue.

MATERIALS AND METHODS

Virus and cell cultures. ASPV from the third to fifth passages (Kvellestad et al. 2003) was used. Rainbow trout gill (RTgill-W1) cells (Bols et al. 1994, ATCC CRL-2523) were grown at 14°C in Leibovitz L-15 supplemented with 5% (v/v) foetal bovine serum, 2 mM L-glutamine and 24 µg ml−1 gentamicin. Cells were incubated at 14°C after inoculation with virus without removal of the inoculum.

Production of polyclonal anti-ASPV serum in rabbit. Virus for immunization of rabbit was propagated and purified by CsCl gradient as previously described (Kvellestad et al. 2003) and resuspended in phosphate buffered saline (PBS; 10 mM, pH 7.2). Protein concentration was measured using a commercial assay (Bio-Rad Laboratories) with bovine serum albumin serving as standard. Antigen preparation mixed with Freund’s adjuvant was injected subcutaneously into a rabbit at multiple sites, 3 times at 4 wk intervals. The first injection consisted of 100 µg in complete adjuvant and the last 2 injections of 50 µg in incomplete adjuvant. The rabbit was bled 2 wk after the last injection.

Testing of anti-ASPV serum by IIF staining of ASPV-infected cell cultures. Binding of anti-ASPV serum to ASPV antigen was tested in a cross-titration assay in 96-well microtitre plates essentially as described by Falk & Dannevig (1995). The cultures were inoculated with different virus dilutions, incubated for 7 or 14 d, assessed for cytopathic effects (CPE) by phase-contrast light microscopy, washed in PBS, fixed for 20 min in 80% (v/v) aqueous acetone, air dried, blocked for 30 min with 5% (w/v) non-fat dry milk in PBS, incubated for 1 h with anti-ASPV serum dilutions (2-fold serial in PBS, 1:200 to 1:25 600), and incubated for 1 h with fluorescein isothiocyanate conjugated goat antiserum against rabbit IgG (Southern Biotechnology Associates) diluted 1:50 in PBS with 0.5% non-fat dry milk. PBS with 0.1% (v/v) Tween 20 was used for washing before and after adding secondary antibody. Propidium iodide was added for nuclear staining, and all incubations were performed at room temperature. The cultures were examined using an inverted epifluorescent microscope (Leitz DMIL) with a 50 W mercury lamp. Controls included staining with anti-ASPV serum applied to non-infected cells, and preserum applied to infected cells with CPE, both 2-fold serially diluted 1:25 to 1:3200.

Characterization of anti-ASPV serum by virus neutralization. Anti-ASPV serum inactivated at 45°C for 30 min was tested for the ability to neutralize ASPV. Dilutions of serum (5-fold serial) and virus (2-fold serial) in cell culture medium were mixed to obtain all combinations of final serum dilutions of 5 × 10−2 to 5 × 10−5 and virus concentrations of 10−27, 10−29 and 10−33 TCID50 ml−1. The mixtures were incubated for 2 h 20 min at room temperature, followed by inoculation into 4 replicates of RTgill-W1 cells in 96-well microtitre plates. CPE were assayed by phase-contrast light microscopy at 48 h post inoculation (p.i.).

Characterization of immune serum by haemagglutination inhibition testing. Detection of haemagglutinating activity units (HAU ml−1) was carried out as previously described by Falk et al. (1997). The haemagglutination inhibition test was performed by first mixing 25 µl of anti-ASPV serum (2-fold serially diluted in PBS, 1 × 10−1 to 9.8 × 10−3) with 25 µl virus suspension containing 160 HAU ml−1, followed by incubation for 1 h. Fifty µl of 0.5% (v/v) chicken red blood cells in PBS was then added and the agglutination endpoint read after 1 h of incubation. All incubations were performed at room temperature.

Field sampling of fish gills. Gills were sampled from Atlantic salmon transferred the previous spring to netpens in 3 seawater farms on the western coast of Norway. Farms 1 and 2 were situated in brackish water and Farm 3 in full-strength seawater.

Farm 1 harboured fish of 3 different generations, and during the last week of July a loss of appetite in all groups and a minor increase in mortality in a netpen with fish at 2.5 kg was reported. Two weeks later fish in the postsmolt group displayed signs of respiratory distress, and the cumulative mortality reached 40% over the next 3 mo. The most common gross pathology identified among postsmolt consisted of swollen, grayish-mucinous gills, sometimes combined with a pale liver with scattered bloody spots. Histological examination of gills, heart, liver, pancreas and pyloric ceca,
killed with a blow to the head and exsanguinated. Parallel samples of gill tissue from each fish were fixed in formalin (10% (v/v) in 135 mM sodium phosphate buffer, pH 7.0) or frozen on dry ice and stored at –70°C.

**Histology and IHC.** Gills fixed in formalin for 3 d or more were dehydrated in graded ethanol, cleared in xylene and embedded in paraffin, according to standard protocols. Approximately 5 μm thick sections of gill filaments and lamellae were heated on slides at 60°C for 20 min, dewaxed in xylene and rehydrated through graded ethanols. Rehydrated sections were stained with haemalum and eosin for the study of ferric iron (Stevens 1990), and (on poly-L-lysine coated slides) stained with haemalum and eosin for the study of gill histological changes and mortality.

In all farms, the gills were sampled from fish with clinical signs and weighing 90 to 540 g. The fish were killed with a blow to the head and exsanguinated. Parallel samples of gill tissue from each fish were fixed in formalin (10% (v/v) in 135 mM sodium phosphate buffer, pH 7.0) or frozen on dry ice and stored at –70°C.

**IIF staining of cryosections of gills.** Cryosections of gills stored at –70°C were fixed in 100% acetone for 10 min and IIF stained with preserum (diluted 1:1000 and 1:2000) and anti-ASPV serum (2-fold serially diluted 1:1000 to 1:256,000), according to the protocol described above for cultured cells, but without nuclear staining.

**Results**

**Testing and characterization of anti-ASPV serum**

Binding of anti-ASPV serum to ASPV antigens was demonstrated. IIF staining of ASPV-infected cell cultures incubated for 7 and 14 d p.i., in which CPE were detected by phase-contrast light microscopy in wells infected with the highest virus doses, showed fluorescence from the cytoplasm of cells in all infected wells and with all dilutions of anti-ASPV serum applied (Fig. 1a–c). The intensity of the fluorescent reaction and the number of fluorescing cells decreased with decreasing inoculation dose of virus, and in wells in-
occulated with the lowest doses the positive cells were observed in clusters surrounded by large areas of cells showing no fluorescence. Fluorescence from nuclei was never observed. The anti-ASPV serum could be diluted up to 1:6400 without any significant loss in fluorescence intensity from cells incubated for 7 d. A slight diffuse unspecific cytoplasmic fluorescence was present in uninfected cells up to 1:3200 dilution of anti-ASPV serum. No fluorescence was observed in infected cells to which preserum was applied.
Anti-ASPV serum dilutions up to 1:10 240 and 1:5120 neutralized virus infectivity at titres of $10^{2.7}$ and $10^{2.9}$ to $10^{3.3}$ TCID$_{50}$ ml$^{-1}$, respectively. Serum dilutions up to 1:2560 inhibited the agglutination of chicken erythrocytes by a virus amount of 80 HAU ml$^{-1}$. The results indicate binding of anti-ASPV serum to the haemagglutinin present at the surface of virions and infected cells.

Pathology

Pathologic changes of the same type (Fig. 2) were detected in gills of all fish from the 3 farms, although with great individual variation. Briefly, the gills were macroscopically pale. Histologically, erythrocytes and inflammatory cells were seen inside and/or outside the central venous sinus of the filaments. The latter included macrophages phagocytesing erythrocytes and accumulating ferric iron, as demonstrated by Perl’s Prussian blue reaction.

Lamellar vascular changes included blood accumulation in lumina and/or haemorrhages and/or thrombosis and death of endothelial (pillar) cells. In the epithelium we observed death of epithelial cells, proliferation of apparently poorly differentiated epithelial cells especially in the interlamellar epithelium, and infiltration with inflammatory cells. In some lamellae there was a clearly positive correlation between organization of thrombi and epithelial hyperplasia. Ferric iron was also demonstrated in macrophages phagocytosing erythrocytes in the lamellar epithelium. Varying numbers of epitheliocysts, trichodinids and *Ichthyobodo necator*-like parasites were also seen. The changes occurred segmentally or along the whole length of the filaments, and the lamellar changes frequently resulted in thickening of the apical margin.

Indirect immunofluorescence staining of tissue sections

The gills of 1 fish (Fish 335, Farm 1) with few epitheliocysts, but extensive vascular changes presumptively induced by viral infection were selected in order to test anti-ASPV serum.

The preservation of tissue morphology was not optimal, but fluorescence was observed in the cytoplasm of a moderate number of lamellar cells, apparently both in the epithelium and in the vascular system (Fig. 1d,e), and in a very few cells apparently located in the central venous sinus endothelium. The majority of fluorescing cells were clustered within single lamellae or groups of lamellae. The fluorescence was either homogenous from all parts of the cytoplasm, was partly granular, or occurred from a few very small granules. Staining of nuclei was never observed. Non-specific fluorescence was eliminated by dilution of anti-ASPV serum to 1:1000. Specific cytoplasmic fluorescence was extensive, moderate and slight at dilutions up to 1:8000, 1:32 000 and 1:64 000, respectively, and not observed at higher dilutions. No fluorescence was observed in sections stained using preserum as primary antibody.

Gills from other individuals and with histological changes suspected to have been induced by viral infection were then stained with anti-ASPV serum diluted 1:2000 and with sections from Fish 335 used as controls. Preserum and anti-ASPV serum were applied to the control sections. Weaker specific fluorescence as described above for Fish 335 was observed in gills from a number of these fish (Table 1).

Immunohistochemistry

The anti-ASPV serum gave strong reactions in the cytoplasm of lamellar endothelial and epithelial cells, and possibly also in inflammatory cells in the epithelium of many lamellae when applied to sections from Fish 335 (Fig. 1f–j). The reaction became slight when the anti-ASPV serum was diluted 1:10 000 and 1:100 000 and absent at higher dilutions. The preserum gave no reaction.

Gills from all fish examined by IIF were then examined by IHC. Gills from Fish 335 were used as controls. Fish displaying positive reactions in gills with pathologic changes as described above were seen in all farms (Table 1). In a few fish, staining of few endothelial cells of the central venous sinus was also observed. Individual unspecific reactions varied from slight to moderate, and were seen especially in chloride cells, indicating reactions to mitochondria. Apparently, no correlation existed between the detection of ASPV by immunostaining and epitheliocysts by haemalum and eosin staining, as none, one or both agents were detected in one and the same fish.

No cross-reactions were observed, as specific antibodies to ASPV, VHSV, IHNV, IPNV and ISAV reacted with homologous virus only.

DISCUSSION

In this study we have briefly described the pathological gill changes of PGI in seawater-farmed Atlantic salmon in Norway. The changes described, i.e. circulatory disturbances, inflammation, cell death and proliferation, are changes that may occur individually in gills for several reasons (Ferguson 1989), but the combined presentation of these observed changes is, to the best of our knowledge, different from other gill dis-
PGI may therefore be regarded as a distinct disease. ASPV was detected by immunostaining of epithelial and endothelial cells of pathologically altered tissues, and not found in apparently normal tissues. This association of ASPV with tissue damage demonstrates that the virus is at least a contributing cause of the disease.

Further, ASPV was neither detected in the gills of all the fish nor at all locations with gill vascular and epithelial changes, indicating that the detection meth-

Fig. 2. *Salmo salar* L. Atlantic salmon with proliferative gill inflammation (PGI). Haemalum and eosin stained section of formalin-fixed and paraffin-embedded gill. (a) ×50, (b–g) ×305. (a) One filament with extensive proliferation as the most prominent change in its distal part (right) and many thickened lamellae in the proximal part (left). (b) Lamellae with extensive erythrocyte accumulation in capillary lumina. (c) Lamellae with haemorrhages. Few inflammatory cells were present in the epithelium (arrowhead) and centrally in the filament (arrow). (d) Lamellae with death of endothelial (pillar) cells and thrombosis (long arrow), and basally in epithelium oedema (asterisk) and inflammatory cells (arrowhead). A few epitheliocystis inclusions in 1 lamella (short arrows). (e) Two previous lamellae with apparent disappearance of vascular system above the positions of the arrows. Extensive epithelial proliferation (diamond), remnants of many necrotic cells (asterisk) and inflammatory cells in distended lumen of the marginal channel in a lamella (arrowhead). (f) Epithelial proliferation especially in interlamellar epithelium (diamond) and along lamellae where inflammatory cells are also present.
Table 1. *Salmo salar* L. Atlantic salmon paramyxovirus (ASPV) and epitheliocystis inclusions detected in gills of *S. salar* with proliferative gill inflammation (PGI). Gills from selected fish were examined in parallel by indirect immunofluorescence (IIF) on cryosections, histology and immunohistochemistry (IHC) on sections from formalin-fixed and paraffin-embedded tissue.

<table>
<thead>
<tr>
<th>Farm</th>
<th>No. of fish examined</th>
<th>No. of fish in which ASPV was detected by immunostaining*</th>
<th>No. of fish in which epitheliocysts were detected by histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>5 (IIF: 2, IHC: 5)</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>5 (IIF: 5, IHC: 2)</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>27</td>
<td>12 (IIF: 11, IHC: 1)</td>
<td>11</td>
</tr>
</tbody>
</table>

*Total number (number detected by methods indicated)*

The pathological changes result in impaired gill function due to reduced surface area, impeded blood flow, increased distance between blood and water, and loss of cells necessary for gill function (Ferguson 1989, Roberts & Rodger 2001). The extensive inflammation also indicates an increased activity of haematopoietic tissue.

Mean water temperatures ranging from 17°C in August to 12°C in September in Farm 1 were similar to those in Farms 2 and 3 and are within the temperature range 6 to 21°C observed for *in vitro* replication of ASPV. The slow *in vitro* replication rate observed for ASPV may also explain the long duration of disease outbreaks observed in the fish farms. The occurrence of virus in association with acute vascular changes, i.e. haemorrhage and thrombosis, and with chronic reactive changes of the epithelium, i.e. hyperplasia and infiltration with inflammatory cells, in one and the same fish also indicate a protracted disease course at the individual level.

To the best of our knowledge, this is the first report of a fish disease related to infection with a member of the *Paramyxoviridae*. Isolations of paramyxovirus-like viruses from chinook salmon (Winton et al. 1985) and carp (Body et al. 2000) also indicate that this virus family is represented among fish pathogens. The detection of ASPV in epithelial and endothelial cells is consistent with the properties of a number of paramyxoviruses that can initiate systemic infection following primary replication in the respiratory mucosa of man and of warm-blooded animals (Tyler & Nathanson 2001).

In conclusion, ASPV has been detected by cell culture isolation and by immunostaining in gills of Atlantic salmon with PGI. It is therefore concluded that ASPV is at least a contributing cause of PGI, and its significance as an aetiological agent of PGI and possibly other diseases of unsolved aetiology should be further investigated. The apparent multifactorial aetiology of PGI recommends an epidemiological approach including the use of more sensitive methods to detect ASPV and chlamydia-like bacteria in tissues. An infectivity challenge model with ASPV should also be established for the study of pathogenesis, especially to clarify whether the gills are the primary site of replication, and to sequentially describe the development of pathological changes in gills and possibly also other organs.

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