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**Immunohistochemical localization of inflammatory cells and cell cycle proteins in the gills of *Loma salmonae* infected rainbow trout (*Oncorhynchus mykiss*).**

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Running title: Immunohistochemistry of *Loma* infected gills

**Abstract**

Microsporidial gill diseases particularly those caused by *Loma salmonae* incur significant economic losses to the salmonid aquaculture industry. The gill responses to infection include the formation of xenomas and the acute hyperplastic inflammatory responses once the xenomas rupture releasing infective spores. The aim of this work was to characterize the inflammatory responses of the gill to both the presence of the xenomas as well as the hyperplasia associated with *Loma salmonae* infection in the rainbow trout gill following an experimental infection using immunohistochemistry. Hyperplastic lesions demonstrated numerous cells expressing PCNA as well as an apparent increased expression of caspase-3 and number of apoptotic cells (TUNEL positive cells). There was expression of TNFα in individual cells within the gill and increased expression of a myeloid cell line antigen indicating the presence of granulocyte infiltration of both the hyperplastic lesions as well as the xenomas. Similar immune-reactivity was seen in gill EGCs. Hyperplastic gill lesions showed a marked infiltration of CD8+ cells and expression of MHC class I antigens. These findings suggest that *Loma salmonae* xenomas may be subject to infiltration by the host immune cells as well as the mounting or a marked cellular cytotoxic immunoreaction in the resultant hyperplasia following xenoma rupture and spore release.

**Key words:** microsporidial gill disease; xenoma; proliferating cell nuclear antigen; apoptosis; granulocyte; CD8; MHC I

## 1. Introduction

Microsporidial gill diseases (MGDs) pose significant limitations to the development of marine fish aquaculture specifically in the North Atlantic region [1-2], North Pacific [3] and recently red sea [4]. *Loma salmonae* infections cause significant economic losses to salmonid aquaculture affecting both hatchery [5-6] and marine production [7-8]. The naïve host ingests the *Loma salmonae* spores that enter the host through the digestive tract, where the sporoplasm is injected into a host cell that then migrates to the heart for a 2 week merogony-like phase followed by a macrophage-mediated transport to the gill where endothelial and pillar cells are hypertrophied to form xenomas containing microsporidial spores [9-11]. Microsporidian xenomas are visible on the gills characterized by their distinct morphology with the *Loma* sp. having a well defined and characteristic thick granular amorphous wall with various developmental stages being present throughout the xenoma [12]. Infection and xenoma formation is temperature and infection method dependent [13-18] with suppressed growth rates, increased metabolic costs and ionic disturbances occurring as a result of ionoregulatory dysfunction 4-10 weeks post exposure [19-21].

Morphologically, the rupture of the xenoma is accompanied by an extensive inflammatory response consisting of filament hyperplasia, neutrophil and macrophage infiltration and in more severe cases neovascularisation and thrombocytic infiltration [22-24]. Associated with the lesions are also large numbers of dendritic-like cells [25] indicating potential for MHC class II responses underpinning and strong immunological response as is seen with this disease [26].

However, other than morphology, limited description of the inflammatory and immune cell involvement in *Loma salmonae* infections in gills have been described.

Proliferative cell nuclear antigen (PCNA) is a common marker for cell proliferation and surrogate marker for DNA synthesis reflecting cells in different phases of the cell cycle, DNA repair and apoptosis [27]. In proliferating gill tissue, anti-PCNA immune-reactivity has been demonstrated with other gill diseases such as amoebic gill disease of Atlantic salmon [28]. Caspases (cysteine-dependent aspartate protease) are key players in the process of apoptosis as well as necrosis and inflammation [29]. Taken together, TUNEL (terminal deoxynucleotidyl transferase nick-end labeling) and caspase 3 immuno-staining have been suggested as the best approaches to detect apoptosis in some conditions such as heart failure [30]. Apoptosis is often associated with oxidative stress of which HIF1α is a primary marker and key transcription factor [31] that can also be activated by cytokines such as TNFα [32-34]. Innate cellular immune responses include the actions of granulocytes, mast cells, dendritic cells and natural killer cells [32] which the adaptive cellular immune responses comprise of the recognition of cell surface MHC-peptide complexes including MHC class I and II by T lymphocytes including CD4+ and CD8+ cells.

The aim of this study was therefore to investigate the expression of cell-cycle regulatory proteins (PCNA, HIF1α), apoptosis (Caspase 3, TUNEL), and immune-inflammatory responses (TNFα, myeloid-like cell granulocyte antigen, CD8 and MHC class I) in rainbow trout gills infected with *Loma salmonae*.

## 2. Materials and Methods

### *2.1 Fish husbandry and experimental infection*

Juvenile rainbow trout (*Oncorhynchus mykiss*) of mean mass and fork length (± SEM) of 20.7 ± 0.8 g and 13.3 ± 0.2 cm respectively were obtained a certified specific pathogen free commercial hatchery with no history of *Loma salmonae* and transferred to the Atlantic Veterinary College, University of Prince Edward Island as described by Powell et al. [21]. Fish were randomly allocated to three, 70 L flow-through round fibreglass tanks at a flow rate of 2 L $min^{-1}$ and temperature 15$^{o}$C and fed daily at 2% body weight on a commercial diet (HiPro 3.0 GR; Corey Feed Mills, Fredricton, NB, Canada). All procedures were in accordance with the Canadian Council on Animal Care (1993). As described by Powell et al. [21], fish were fed *Loma salmonae*-infected macerated gill material (with a minimum estimate of $2x10^6$ spores per tank obtained from laboratory fish maintained with a *Loma salmonae* infection. Fish were maintained and monitored for 5 weeks until the peak number of xenomas had occurred [21] after which 5 animals that had been infected with *Loma salmonae* and 5 uninfected controls were removed, killed with an overdose of benzocaine (> 60 mg $L^{-1}$) and the gills removed and fixed in neutral buffered formalin.

### *2.2 Histology and immunohistochemistry*

Gill tissue was dehydrated through a graded series of alcohols and xylene and embedded in paraffin wax before sectioning at 0.5 µm. Sections were stained with haematoxylin and eosin. Archived paraffin wax histology blocks were used for the present immunohistochemistry study

where sections were re-cut and stained as described below. Antibodies used in this study included polyclonal rabbit proliferative cell nuclear antigen (PCNA, sc-7907, Santa Cruz Biotechnology, Santa Cruz, CA , USA) 1:150, polyclonal rabbit caspase 3 (sc-7148, Santa Cruz Biotechnology) 1:500, and polyclonal goat hypoxia inducible factor (HIF1α, Santa Cruz Biotechnology) 1:200. Additionally, polyclonal rabbit anti-trout TNFα antiserum [35] 1:500, mouse monoclonal anti-trout granulocyte antibody recognizing myeloid-cell line derived cells (MCG) 1:2000, monoclonal mouse anti-salmon antibodies to CD8 αF1-29 and MHCIαF1-34 (MHC I) [36-38] were used. All non-salmonid antibodies have shown specificity to salmonid tissues by western blot [39].

Immunohistochemistry followed the protocol described by Haugarvoll et al. [40]. Sections were mounted on poly-L-lysine (Sigma Aldrich Norway) coated slides and dried at 50$^{o}$C for 30 min. Sections were then deparaffinised in xylene (3 x 5 min) followed by rehydration through a series of graded ethanol baths to distilled water. Antigen retrieval involved autoclaving the slides at 121$^{o}$C for 10 min in 10 mM citrate buffer, pH 6.0 containing 0.1% Tween 20 (Sigma Aldrich Norway). Slides were cooled to room temperature then washed twice with de-ionised water (2 min each). All incubations were performed in a closed-lid humidity chamber. The sections were incubated in 5% bovine serum albumin (BSA, Sigma-Aldrich Norway) in phosphate buffered saline (PBS) for 1 h, then incubated with primary antibodies in 1.5% BSA in PBS overnight at 4$^{o}$C. Following incubation, slides were washed with PBS with 0.1% Tween 20, pH 7.4 (3 x 5 min) then incubated with secondary antibodies. Secondary antibodies were conjugated to horseradish peroxidase (HRP) (sc-2020, donkey anti-goat or sc-3837 goat anti-rabbit, Santa Cruz

Biotechnology, California USA) in 1.5% BSA in PBS for 60 min at room temperature. Following incubation slides were washed with PBS containing Tween 20 as above and to inhibit endogenous peroxidase activity, slides were incubated with 3% hydrogen peroxide (Sigma Aldrich Norway) in ethanol for 10 min at room temperature. Slides were rinsed with PBS containing Tween 20 then incubated with 3,3'-diamniobenzidine (DAB, Sigma-Aldrich Norway). Slides were counterstained with haematoxylin, passed through a graded series of ethanol and xylene and mounted with poly-vinyl-alcohol (PVA) mounting media, pH 8.2 (Histolab, Oslo, Norway). Negative controls of both un-infected and infected gill tissue section consisted of replacement of the primary antibody with 1.5% BSA in PBS.

Immunohistochemistry using anti-salmon CD8 and MHC class I mouse monoclonal antibodies was performed using the Tyramide Signal Amplification (TSA) Biotin system (Perkin Elmer/NEN Life Science, Boston, MA, USA). The protocol was as described above until antigen retrieval then slides were incubated with 3% hydrogen peroxide in ethanol (10 min) then washed with PBS. Sections were blocked with blocking buffer (0.1M Tris-HCl, pH 7.5, 0.14 M NaCl, 0.5% blocking agent supplied with the TSA system) for 1 h then slides were incubated with primary antibodies as above overnight then washed with PBS (3 x 5 min). The slides were then incubated with HRP-conjugated anti-mouse secondary antibody (sc-3697, goat anti-mouse, Santa Cruz Biotechnology), in blocking buffer for 1 h, then washed in PBS (3 x 5 min). Slides were treated with biotinyl tyramide amplification agent followed by streptavidin-horseradish peroxidase (SA-HRP, provided with the kit). Slides were then incubated with AEC (3-Amino-9-ethylcarbazole) and counterstained as described above, before being mounted using

ImmunoHistoMount (Sigma-Aldrich, Norway). Negative controls consisted of replacing primary antibodies with TNB blocking buffer and unamplified control that included all of the reagents except TSA reagents. Both control (un-infected) and infected tissue sections were used for negative controls.

*2.3 TUNEL staining*

To identify apoptotic cells, Terminal deoxynucleotidyl Transferase (TdT) Biotin-dUTP Nick End Labeling (TUNEL) was performed using ApopTag$^{TM}$ Plus Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon$^{TM}$ International Inc USA). Deparaffinised and hydrated slides, were treated with freshly made IHC Select$^{TM}$ Proteinase K (20 mg mL$^{-1}$)(Millipore AS Oslo, Norway) for 15 min at room temperature then washed with distilled water($dH_2O$, 2x 2min). Endogenous peroxidase was quenched with 3% hydrogen peroxide in PBS for 5 min at room temperature, the slides rinsed with $dH_2O$, then equilibration buffer (provided in the kit) was applied at 75 µL 5 cm$^{-2}$ for 10s. TdT enzyme was applied at 55 µL 5cm$^{-2}$ and incubated at 37$^{o}$C for 1 h. Slides were dipped in stop/wash buffer, then incubated for 10 min at room temperature before washing in PBS 3 x 1 min). Anti-digoxigenin conjugate was applied at 65 µL 5 cm$^{-2}$ of section and incubated at room temperature for 30 min in a humidified chamber before being washed in PBS (4 x 5 min). Peroxidase substrate was applied at 75 µL 5cm$^{-2}$ and incubated to 3-6 min at room temperature. Sections were washed in $dH_2O$ (3x 1 min) and counterstained with 0.5% methyl green (Sigma-Aldrich, Norway) for 10 min and washed with $dH_2O$ then in 100% N-butanol before dehydration and mounting with PVA based mounting media as above. Positive controls were conducted (provided with the kit) and negative controls were performed by replacing the TdT with

equilibration buffer. Both control (un-infected) and infected tissue sections were used for negative controls.

## 3. Results

Gills of unchallenged fish (controls) showed no signs of disease or parasitism with narrow lamellar and filament epithelium. There were no signs of xenomas nor filament hyperplasia (Fig. 1 A). Challenged fish, on the other hand, demonstrated a number of branchial xenomas, some of which were associated with focal hyperplasia of the filament epithelium (Fig. 1 B-D). In immune-localisation studies, there was a high degree of consistency between the five fish in each group and between examined sections. Negative controls (tissue sections from both uninfected and infected fish) showed little or no non-specific staining (Fig. 2 and Fig. 4) whereas positive staining reactions were clearly seen in sections in nuclei or cellular cytoplasm depending upon the antibody being used. Proliferating nuclear antigen positive cells were evident in the filament and lamellar epithelium of unparasitized gills (Fig. 2A). Positive staining epithelial cells were small and squamous. In *Loma salmonae* infected gills, the levels of staining appeared more extensive with numerous cells in the filament epithelium staining PCNA positive (Fig. 2C). Positive staining cells were also evident in the epitheliod cells surrounding xenomas and within the hyperplastic lesions associated with *Loma salmonae* infection (Fig. 2C and D). Within the lesions, numerous rounded cells stained PCNA positive (Fig. 2D). Caspase 3 positive staining cells were observed in filament and lamellar epithelium of unparasitised (control) trout gills (Fig. 2E) where as in parasitized gills, caspase 3 positive cells were more confined to the filament epithelium (Fig. 2F). Xenomas also showed positive immune-staining for caspase 3 (Fig.

2F). In unparasitised (control) gills, HIF1α positive e cells were located on the filamental and lamellar epithelium consisting of weak positive staining of squamous cells and more intense staining of rounded cells (Fig. 2G), a similar pattern of staining was observed in *Loma salmonae* infected gills with HIF1α positive cells confined to the epithelial edge of branchial hyperplastic lesions (Fig. 2H).

Tumor necrosis factor α positive cells were rarely observed in the gills of unparasitised (control) gills (not shown) and positive staining was observed in a few cells within the hyperplastic lesion associated with *Loma salmonae* infection (Fig. 3A) and in cells infiltrating branchial xenomas (Fig. 2B). Positive TNFα immune-reactivity was observed in eosinophilic granule cells associated with the filament and central venous sinus (Fig. 3A). Positive immune-reactivity was observed for the MCG antigen in unparasitized (control) gills where few positive cells were located primarily within the filament epithelium (Fig. 3C). In *Loma salmonae* infected gills, MCG positive cells were noted surrounding and penetrating the xenomas (Fig. 3D) and infiltrating the hyperplastic lesions associated with infection (Fig. 3E). Positive immune-reactivity was also observed in EGCs (Fig. 3D and E). Few CD8 positive cells were observed in un parasitized (control) gills (not shown) although *Loma salmonae* infected fish showed pronounced positive staining in the filament epithelium and hyperplastic lesions with cytoplasmic staining also discernable in some cells (Fig. 3F). Although there was occasional staining of MHC class I positive cells in the gills of unparasitised controls (not shown), there was marked infiltration of the filament epithelium and hyperplastic lesions of Loma *salmonae* infected gills (Fig. 3G).

Immuno-reactively positive cells were rounded and scattered throughout the hyperplastic lesions.

TUNEL positive cells were seen scattered throughout the filament and lamellar epithelium of un parasitized controls (Fig. 4 A) and in the gills of *Loma salmonae* infected fish (Fig. 4 C and D) with occasional xenomas showing evidence of positive cells in the surrounding epithelial cells and within the xenoma itself (Fig. 4C). However, there appeared to be no differences in the apparent number of cells showing TUNEL positive staining between controls and parasitized gills.

## 4. Discussion

Healthy and non-parasitized rainbow trout gill tissue has a relatively high cell turnover as evidenced by the levels of immunoreactivity using PCNA, caspase 3 and by TUNEL labeling. However, the presence of *Loma salmonae* xenomas and the hyperplastic responses to *Loma salmonae* infections appeared to enhance the degree of immunoreactivity of PCNA with cells staining positively within the hyperplastic lesions, as well as cells surrounding the xenomas. Increases in PCNA activity has been shown in other parastized gill models such as with amoebic gill disease of salmon [28] where increased both gene expression and immunoreactivity was demonstrated in lesioned areas of the gill. Increased levels of caspase 3 positive staining may be indicative of the initiation of apoptosis [29, 41]. The antibody used for detection of caspase 3 in the present study is specific for caspase 3 in fish as confirmed by western blot analysis [39]. Of note was the presence of positive caspase 3 immunostaining within the xenomas as well as

cells within the filament epithelium. This staining appeared to be specific (not evident in controls) and suggests the initiation of cell death processes within the xenoma, most likely through the intrinsic activation pathway [29]. The absence of large numbers of TNFα immunopositive cells suggests that the extrinsic pathway for apoptosis is unlikely to be the primary mechanism in *Loma*-affected trout gills [29].

In association with increased caspase 3 expression there was an apparent increase in number of TUNEL positive cells within branchial lesions, is strongly suggestive of an increase in the apoptotic rate and thus increased cell turnover. As with caspase 3 immunoreactivity, there was evidence of TUNEL positivity within the xenomas although at a lower level compared with that of caspase 3. The significance of this is unclear but suggests the initiation of apoptotic processes of cellular tissue within the xenomas. The identity of the cells expressing caspase 3 and TUNEL immunoreactivity was not discernible and should be investigated further. The expression of HIF1α immunoreactivity was primarily confined to occasional epithelial cells including those on the periphery of hyperplastic lesions. The significance of this is not clear although TNFα released during infections has been suggested to activate HIF1 in mammals [34] and has been suggested as an explanation for the expression profile of HIF1α in HSMI, CMS and PD affected Atlantic salmon hearts [39]. However, in the gill, TNFα expression in *Loma salmonae* infected gills was low with only occasional cells within the filament epithelium staining positive although these included cells within hyperplastic tissue as well as associated with the branchial xenomas, none of which coincided with the expression profile of HIF1α. In Atlantic salmon, the tTNFα antibody binds to a constitutively expressed 60 kDa protein [28]. However, even when Atlantic

salmon were significantly affected by amoebic gill disease TNFα expression was not increased confirming earlier findings by the same group [42] that TNFα upregulation was probably not an intrinsic feature of AGD pathophysiology [28]. In the present study, anti-TNFα did stain positively granulocytes within the gill around the central venous sinus. The location and morphology of these cells suggests them as eosinophilic granular cells (EGC). This observation supports similar immunoreactivity of this cell-type in the heart [39]. The expression of TNFα in EGCs is consistent with their putative role as mast cells [43]. Mast cells are known to release TNFα to enhance T cell activation [44]. Indeed TNFα also enhances EGC recruitment in conjunction with LPS [45]. However, the low levels of TNFα expression in the *Loma salmonae* infected rainbow trout gill and no evidence of EGC hyperplasia or proliferation in the gill, suggests that this may not be important with this parasitic infection. The same EGC cells were stained positively with anti-granulocyte marker (MCG) consistent to the application of this antibody in Atlantic salmon with virally induced cardiac pathologies [39]. Other granulaocyte-like cells were also detected within the hyperplastic lesions in significant numbers consistent with the ultrastructural observations of neutrophilic infiltration of the gill hyperplasia in *Loma salmonae* inffected fish [24-25]. The marked increase in numbers of CD8 positive cells in the hyperplastic lesions indicated the involvement of cytotoxic T cells in the immunoreaction to *Loma salmonae* in trout. In addition the strong levels of MHC class I staining in *Loma*-affected hyperplastic lesions is consistent with suggestions that MHC class I antigen expression is ubiquitous [46]. Increased numbers of MHC class I positive cells in the lesions is indicative of antigen presentation within the hyperplasic tissue, most probably by macrophages as suggested in the heart using the same antibody [39].

It was apparent that it was clearly possible to perform immunohistochemistry on rainbow trout gill tissue from archived wax histology blocks with little apparent evident of loss of antigenicity or tissue degradation. That notwithstanding, the degree to which cellular degradation or antigenicity was compromised was not assessed. Quantitative measures of responses in this study was not possible given that only a limited number of blocks could be used in the study where sufficient tissue remained to demonstrate histological lesions and xenomas infected fish. Similarly, quantitation of immunoreactive tissue is limited by the definition of controls. A negative reaction may not always indicate absence of a given antigen, just poor or non-specific staining. In the present study, qualitative histological assessments were made to avoid these potential difficulties.

In conclusion, within *Loma salmonae* induced hyperplastic lesions in rainbow trout gills, there appears to be marked expression of PCNA and, albeit to a lesser extent, some caspase 3 activity although evidence of apoptosis by TUNEL staining was marginal. This indicated an actively dividing tissue with the infiltration of granulocytic cells CD8 positive lymphocyte-like cells in association with a strong expression of MHC class I antigen. In addition, the expression of TNFα positive cells and MCG positive cells within the branchial xenomas indicated penetration and infiltration of the xenomas by host immune cells prior to the onset of filamental epithlial hyperplasia. These responses represent the tissue reaction in rainbow trout, a model species for which *Loma salmonae* infections are rarely lethal and a strong immune-competence develops in recovered fish [26]. The pronounce CD8 and MHC response coupled with the strong granulocytic response correlates well with a strong immune-competence. In other susceptible

species of the *Oncorhynchus* genus, [5-7, 22-24], the immune-response may not be as pronounced and the associated tissue reactions would warrant further investigation.

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Figures

Figure 1. Representative histological sections stained with haematoxylin and eosion from unparasitised (control) fish (A), gill filaments with focal hyperplasiatic lesions (arrows) (B), hyperplastic lesion surrounding a xenoma (arrow) with prominent eosinophilic granule cells (arrowhead) (C) and a single xenoma (arrow) in non-hyperplastic gill tissue (D) of *Loma salmonae* infected rainbow trout. A, C, D Bar represents 50 μm, B bar represents 200 μm .

Figure 2. Immunohistochemical localization of proliferating cell nuclear antigen positive cells (arrow) in (A) unparasitized rainbow trout gill and (B) negative control, (C and D) PCNA positive cells (arrow) in interlammaellar hyperplastic lesions and cells surrounding *Loma salmonae* xenoma. (E) caspase 3 positive cells in the epithelium of unparasitised gill (arrow) and (F) filamental epithelium and *Loma salmonae* xenoma (arrows). (G) Hypoxia inducible factor 1α positive cells in the filamental and lamellar epithelium of unparasitized gill (arrows) and (H) hyperplastic lesion associated with *Loma salmonae* infection. A-F and H bar represents 50 μm, G bar represents 100 μm.

Figure 3. Immunohistochemical localization of tumor necrosis factor α positive cells (arrow) in a hyperplastic lesion (A) and *Loma salmonae* xenomas (B) in the gills of rainbow trout. Arrow heads indicate localization of TNFα positive eosinophilic granule cells, EGCs. Myeloid cell line granulocyte positive cells (arrow) in unparasitized rainbow trout gills (C), surrounding and penetrating a *Loma salmonae* xenoma (D) infiltrating a hyperplastic lesion (E) arrow heads indicate localization of antigen in EGCs. Localisation of cytoplasmic staining (inset) of CD8

positive cells (arrows) in the filament epithelium and hyperplastic lesions (F). Localisation of MHC class I positive cells in the filament epithelium and within hyperplastic lesions (arrows) of *Loma salmonae* infected rainbow trout (G and H). A-F and H bar represents 50 μm, G bar represents 100 μm.

Figure 4. Localisation of TUNEL positive cells (arrowed) in unparasitized rainbow trout gill (A) negative control (B), in the filament and lamellar epithelium of gills infected with *Loma salmonae* xenoma (C) and hyperplastic lesion associated with infection (D). A and B bar represents 200 μm, C and D bar represents 100 μm.