

Histopathological study of gills in experimentally amoebic gill disease (AGD) infected Atlantic salmon, *Salmo salar*, L.

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Summary

Amoebic gill disease (AGD) is the most important parasitic disease of Atlantic salmon industry in Australia. Atlantic salmon (*Salmo salar*) experimentally infected with *Neoparamoeba* sp. apparently showed AGD gross signs on the gill and an amoebic-associated gill pathology. Physico-chemical factors of water during the experiment were monitored regularly and were approximately constant (temperature: 17°C, salinity: 35 g/l, total ammonia: 0.25 mg/l, pH = 7.9). In this study significant gill pathology was observed histologically, and in all of the sections a multifocal hyperplasia and fusion of adjacent secondary lamellae was seen. The severity of pathological changes observed in the sections did not always correspond with the number of amoebae and even occurred in the absence of amoebae. Some histopathological changes that were seen in the secondary lamellae are: thickening of the secondary lamellae due to hyperplasia, reduction in chloride cell density and an increase in mucous cell numbers of the epithelium. Some of neighboring secondary lamellae was seen attached to one another, but entire fusion of the primary lamellae was not observed. Amoebae were seen in all sections in significant densities mostly in the outer part of hyperplastic tissues.

Key words: Histopathology, *Neoparamoeba*, Atlantic salmon, Amoebic gill disease

Introduction

Amoebic gill disease (AGD) is an important disease of Atlantic salmon (*Salmo salar*) in Tasmania and has been reported elsewhere in the world (Munday *et al.*, 2001). Morphology of *Neoparamoeba pemaquidensis* and the newly described *N. branchiphila* have been attributed as the causative agent of AGD and been studied extensively by Dykova *et al.*, (2005).

Different aspects of AGD of Atlantic salmon have been extensively studied in the recent years (Zilberg *et al.*, 2000; Parsons *et al.*, 2001; Clark *et al.*, 2003; Powell and Clark, 2003a; Powell and Nowak, 2003; Harris *et al.*, 2004; Roberts and Powell, 2004) with a focus towards improving treatment for the disease. Typical pathological changes of fish infected with *N. pemaquidensis* reported as the hypertrophy and desquamation of surface epithelial cells

within the immediate vicinity of attachment, hyperplasia and thickening of secondary lamellae as well as oedema of the epithelium (Adams and Nowak, 2003). This study was carried out to investigate the pathological changes of gill in Atlantic salmon smolts, following experimental infection with *Neoparamoeba* sp.

Materials and Methods

Amoeba isolation

In order to infect the fishes, amoebae were isolated from AGD-affected Atlantic salmon from an ongoing laboratory infection at University of Tasmania, according to Morrison *et al.*, (2004). Briefly, gills were excised from dead fish and excess blood was rinsed off and the gills were placed in filtered autoclaved seawater. Individual arches were dissected and placed in 50 ml tube with distilled water. After agitation

gently for 20 sec, the tubes were centrifuged at 450 x g for 5 min. The supernatant discarded and the tubes re-filled with filtered seawater. The seawater was poured out to plastic petri dishes and amoebae were allowed to adhere for 1-1.5 hr. To remove adherent cells, 750 µl of trypsin/EDTA (0.05% trypsin, 0.53 mM Na₄EDTA) was added to each petri dish and gently shaken to help dislodge cells. The cells were washed by centrifuging in seawater (450 x g for 5 min) and discarding the supernatant and re-suspending the pellet in seawater (Powell *et al.*, 2003b). For increasing the amoebae number, the cells were incubated overnight at 18.5°C.

Experimental infection of fish

Twenty apparently non-AGD affected Atlantic salmon (total length: 18 ± 3 cm and weight: 80 ± 15 g) were infected with 700,000 isolated amoebae (250 amoebae per litre of water) in a 2750 L recirculation system. Water quality was monitored regularly for temperature, salinity, ammonia, dissolved oxygen and pH during the experiment. These factors were approximately constant during the experiment as follows; temperature: 17°C, salinity: 35 g/l, total ammonia: 0.25 mg/l and pH = 7.9.

Sampling

Sampling commenced immediately before infection (5 fish) and after 10 days post-infection, when gross signs of the disease were appeared (raised white mucoid patches on the gills) (Munday *et al.*, 2001). After euthanizing with approximately 0.2% clove oil, the weight, length and gross gill scores were recorded for each fish. The gills were excised and fixed in seawater Davidson's fixative for 24 hrs and the second left anterior hemibranch was processed for histopathology. Gills of 10 normal fish were also excised and sectioned as a control for comparison.

Histopathology

After 24 hrs of fixing, the gill arches were transferred to 70% ethanol. Each gill arch was dehydrated, embedded in paraffin, sectioned at 5 µm and stained with haematoxylin and eosin (H&E). Each gill

arch was assessed for any lesion using light microscopy.

Results

Atlantic salmon experimentally infected with *Neoparamoeba* sp. showed AGD gross signs in the gills and the amoebic-associated gill pathology. The gross changes of infected gills were presence of raised scattered opaque white mucoid patches upon the gills and excessive mucus production. Significant gill pathology was observed histologically, and in all of the sections hyperplasia and fusion of the secondary lamellae was seen (Figs. 1 and 2). However, the severity of pathological changes observed in the sections was not always corresponding with the number of amoeba. Occasionally pathological changes were observed in the absence of amoebae in the affected part.

Histopathological gill changes observed in this study can be defined as severe, because severe hyperplasia was seen in about three quarters of gill. Some histopathological changes that were seen in the secondary lamellae are: thickening of the secondary lamellae due to hyperplasia, reduction in chloride cell density and an increase in mucous cell numbers of the epithelium. According to the results, the hyperplastic cells of gills were smaller than the similar cells in normal gills. The cell atrophy was recognized qualitatively by comparison of cytoplasm to nucleus ratio in the affected gills with the normal cells in the control group. In the affected gills, nuclei of the hyperplastic cells were more close to each other and the cytoplasm/nucleus ratio seem to be reduced. The atrophy could be seen obviously in all sections in hyperplastic cells (Figs. 2 and 3).

There was evidence of fusion of neighboring lamellar tips, but entire fusion was not observed. Amoebae were seen in all sections in significant densities mostly in the outer part of hyperplastic tissues (Fig. 3). Enlargement and hyperplasia of goblet cells in localized sites of amoebic infection and accumulation of abnormal amounts of mucous material in goblet cells was a dominant lesion. Large vacuoles in the hyperplastic gill lamellae were seen and

Fig. 1: AGD affected Atlantic salmon gill. The amoebae induced hyperplasia of gill lamellar tissues. The primary lamellae (P) are fused to each other (arrow). Tissue is composed mainly of undifferentiated epithelial cells and mucous cells (H) and occupied the space between the secondary lamellae (head arrow), (H&E, ×200)

Fig. 2: Amoebae, with prominent nucleus and vacuolated cytoplasm (arrow), are visible in cavity formed by the adherence of secondary lamellae to each other and inflammatory cell (head arrow) around it, (H&E, ×1000)

Fig. 3: *Neoparamoebae* on the surface of gill filament of infected fish in the outer part of hyperplastic tissues, with prominent nucleus (head arrow) and vacuolated cytoplasm. There is a mucus layer around the amoebae (arrow). Hyperplasia of goblet cells (white arrows) and epithelial cells (black arrows) can be seen (H&E, ×1000)

Fig. 4: *Neoparamoeba* sp. infection has caused strong cellular response in gill resulting in epithelial hyperplasia and AGD vesicle (V). The arrow indicates a *Neoparamoeba* cell attached to the surface and there are some inflammatory cells around it (I), (H&E, ×1000)

sometimes amoebae and inflammatory cells were seen within these large vacuoles.

A heavy inflammatory cells infiltration was not seen in the gill lesions despite the extent of the gill pathology, however a few mononuclear and eosinophilic cells were seen in the infected sites, especially in areas where amoebae were attached (Fig. 4).

Discussion

In this study significant gill pathology was observed, and in all of the sections hyperplasia and fusion of the secondary lamellae was seen in some parts of the gills. Gills typically make up about 50% of the total body surface area and are very thin and delicate structure. Amoeba activity on the cell surface and its secretions may irritate the gill lamellae (amoebae produce extracellular proteases) (Butler and Nowak, 2004) so, the congestion and hyperaemia of gill lamellae may be due to direct effect of parasite on the gill. Many of the changes due to this parasitic disease have been reported previously (Adams and Nowak, 2003, 2004; Adams *et al.*, 2004) and appear to be a compensatory response to keep the gill intact after destruction of lamellar epithelium. This study was carried out to investigate the pathological changes of gill in experimental condition on Atlantic salmon smolts after infection with amoeba. The similar reports are in cage culture condition and on salmon in grow out or adult stages, not in smolt stage. Therefore, the results that, *N. pemaquidensis* can cause AGD in smolt salmon, in experimental condition, with typical pathological changes are new finding for the authors and salmon industry.

The hyperplastic epithelial cells of gills appear atrophic, possibly as a result of a diminished nutrient supply due to the increased diffusion distance associated with the hyperplastic lesion. Alternatively, the cells of the hyperplastic lesion are small undifferentiated parenchymal-type cells derived from the basal region of the gill epithelium. The biochemical mechanisms of this potential atrophy are not very well understood. There is a finely regulated balance between protein synthesis and

degradation in normal cells, and either decreased synthesis or increased degradation or both may cause atrophy. Hormones, particularly glucocorticoids, and prostaglandins influence such protein turnover although the role of either have yet to be studied in AGD-affected gills.

Mucous or goblet cells are present in different parts of the gill filaments, especially at the trailing edges and base of lamellae. These cells are responsible for mucus production, which is increased when the gill is irritated by parasites or other irritant materials (Powell and Perry, 1996; Powell *et al.*, 1998). Hypertrophy of goblet cells may be caused by increased functional demand for mucus, but perhaps due to the limitation of vascular and oxygen supply within the lesion itself, degenerative changes occurred in the cells. These changes include lysis and loss of structural and contractile elements that lead to morphological changes of the cells. Lamellar oedema may result from an impaired blood outflow from the secondary lamellae in the infected sites. Increases in hydrostatic pressure may be important secondary effect of AGD, which may occur following hyperplasia of lamellar cells and reduction in flexibility of blood channels. In order to potentially compensate for areas of reduced oxygen diffusion across the gill, blood pressure and local blood flow may be increased in the gills, thus also increasing the potential for oedema. The hypoxic effect in AGD is well discussed by Powell *et al.*, (2000).

Enlargement and hyperplasia of epithelial and goblet cells in localized sites of amoebic infection can be a diagnostic feature. Also, inflammatory cells infiltration are not frequently present in large numbers in AGD lesions and the absence of large numbers of mononuclear and eosinophilic cells from the infected sites suggest that AGD is a chronic obstructive gill disease rather than resulting from the direct infectious parasitic nature of *Neoparamoeba* sp.

Large vacuoles in the hyperplastic gill lamellae were seen as described by Adams and Nowak (2001), because of secondary lamellae fusion. However, some of the smaller vacuoles may arise in part because

of destruction or apoptosis of the goblet cells.

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