Amoebic gill disease (AGD) in Atlantic salmon (Salmo salar) farmed in Chile

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A R T I C L E   I N F O

Article history:
Received 5 September 2010
Received in revised form 31 October 2010
Accepted 1 November 2010

Keywords:
Atlantic salmon
Amoebic gill disease
Neoparamoeba perurans
Sealice

A B S T R A C T

Between May and November 2007, three marine Atlantic salmon farms around Chiloé Island, Chile, reported mortalities in which affected fish presented with Caligus rogercresseyi infections and gross gill lesions characteristic of amoebic gill disease (AGD). Histological examination of the gills from affected fish confirmed the presence of AGD lesions. Trophozoites possessing one or more endosymbiotic Perkinsela amoeba-like organisms (PLOs) were observed in association with hyperplastic tissue. Further analyses were undertaken using a combination of PCR and in situ hybridization and the trophozoites were identified as Neoparamoeba perurans. Thus, our data indicate that N. perurans is a causal agent of AGD in Chile. However, it is possible that AGD was not the single cause of mortalities in the epizootics investigated here. The exceptionally high level of co-infection with Caligus rogercresseyi (maximum mean intensity 34, prevalence 100%), could have contributed to the production losses.

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1. Introduction

Amoebic gill disease (AGD) is a parasitic condition affecting some species of fish farmed in the marine environment (Munday, 1986; Kent et al., 1988; Dyková et al., 1995). AGD is caused by Neoparamoeba perurans (see Young et al., 2007), which is the confirmed aetiological agent of this disease in cases reported from Australia, Ireland, Japan, New Zealand, Norway, USA, Scotland and Spain (Young et al., 2008a; Crosbie et al., 2010; Steinum et al., 2008; Nylund et al., 2008). AGD is grossly characterised by the presence of multifocal lesions in the gills (Munday et al., 1990; Rodger and McArdle, 1996). The presence of these gross lesions allows presumptive diagnoses of AGD in the areas where AGD is enzootic. The final diagnosis is based on histopathology when amoebae that possess one or more endosymbiotic Perkinsela amoeba-like organisms (PLOs) (Dyková et al., 2003, 2008; Adams and Nowak, 2003) are detected in close association with hyperplastic epithelial-like cells (Dyková and Novoa, 2001). Molecular tools, including PCR and in situ hybridization (ISH) have been developed and used to confirm the identity of the parasite (Young et al., 2007, 2008b). In this study, we applied these new diagnostic tools to samples obtained from Atlantic salmon (Salmo salar) that were presumptively diagnosed with AGD in Chile. Our data show that fish were indeed affected by AGD and that Neoparamoeba perurans was the causal agent. However, fish were also co-infected with Caligus rogercresseyi and a causal relationship between either of the parasites and ongoing mortalities could not be established.

2. Materials and methods

2.1. Epidemiology, clinical signs and fish sampling

Between May and November 2007, three marine Atlantic salmon farms in Chiloé Island, Chile (Fig. 1), reported epizootics in which gross lesions typical of AGD (Adams et al., 2004) were observed in affected fish. Fish were sampled and processed for histopathological examination from three farms over the course of one year. Fish from Farm 1 were sampled in May 2007 (20 fish, 10 per cage), August 2007 (16 fish, 8 per cage) and November 2007 (20 fish, 10 per cage). Fish from Farm 2 were sampled in May 2007 (12 fish, 6 per cage), August 2007 (12 fish, 6 per cage) and November 2007 (12 fish, 6 per cage). Salmon from Farm 3 were sampled in August 2007 (10 fish from 1 cage) and November 2007 (31 fish from 3 cages). Fish that displayed signs of lethargy, respiratory stress or surface swimming were targeted for sampling. In addition to histology, all these fish were sampled to confirm the presence of common pathogens in the region, including Piscirickettsia salmonis, Vibrio ordalli and Infectious Pancreatic Necrosis Virus (IPNV). To detect Vibrio spp., samples from...
Fig. 1. A map of Chiloé Island, Chile. The three affected salmon farms are shown on the map.
anterior kidney were cultured on Tryptone-Soy Agar (TSA) (Difco) supplemented with 1% NaCl and then incubated at 20 °C for 3 days. To detect *P. salmonis* samples from kidney were cultured in the CHSE-214 cell line (without antibiotics added) in the presence of neutralizing anti-IPNV antibodies and incubated at 15–18 °C for 28 days (Lannan and Fryer, 1991). The presence of IPNV was determined by culturing CHSE-214 cells in the presence of extracts from the kidneys, spleens and livers from affected fish (Agius et al., 1982). Additionally, reverse transcription PCR (RT-PCR) of RNA from anterior kidney tissues was used to amplify a *P. salmonis*-specific amplicon (Mauel et al., 1996) was used to determine if the fish were positive for *P. salmonis*.

Forty fish from each farm were monitored for sea lice infection every two weeks. The intensity of the infection with the copepod, *C. rogercresseyi*, was quantified as intensity and prevalence of infection (Bush et al., 1997). Fish had been treated for *Caligus* infections using Alphamax (PHARMAQ) in September 2007 on Farm 1 and in August 2007 on Farms 2 and 3.

From July 2007 compulsory quarterly ISA testing was implemented on all salmon farms in Chile (minimum 30 fish, Sernapesca, National Fisheries Service, Chile). Moribund individuals were sampled and analyzed by ISAV-specific RT-PCR (Devold et al., 2000; Mjaaland et al., 2002).

### 2.2. Environmental conditions

Salinity (at 10 m depth), dissolved oxygen concentration (at 0 and 10 m depth) and temperature were monitored during this study. Water quality was measured inside cages on farms at the time of sample collection. Information on rainfall was provided by Department of Hydrology, Sub-department of Meteorology and Snow, Ministry of Public Works (Chile).

### Table 1

Environmental conditions during the AGD outbreak on salmon farms on Chiloe Island, Chile in 2007. Monthly means are shown for salinity, dissolved oxygen and temperature and monthly total is shown for rainfall. Salinity and dissolved oxygen were measured at 10 m depth. Average rainfall for 1993–2006 is also included. Rainfall information was provided by Department of Hydrology, Sub-department of Meteorology and Snow.

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<th>Month</th>
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<th>Average temperature (°C)</th>
<th>Monthly rainfall (mm) (1993–2006)</th>
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### Table 2

Monthly mortality, AGD prevalence and intensity of *Caligus rogercresseyi* infection in Atlantic salmon at seawater farms with outbreaks of amoebic gill disease (AGD). Prevalence of AGD was determined on the basis of histology. At all farms, the prevalence of *C. rogercresseyi* infection was 100% at all times. NA - data not available.

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<tr>
<th>Month</th>
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<th>Farm 1 AGD prevalence (%)</th>
<th>Farm 1 monthly intensity (Caligus/fish)</th>
<th>Farm 2 monthly mortality (%)</th>
<th>Farm 2 AGD prevalence (%)</th>
<th>Farm 2 monthly intensity (Caligus/fish)</th>
<th>Farm 3 monthly mortality (%)</th>
<th>Farm 3 AGD prevalence (%)</th>
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<td>75</td>
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<td>34.0</td>
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Fig. 2. Clinical symptoms of amoebic gill disease affecting Atlantic salmon cultured in Chile. (A) Gross morphology of Atlantic salmon gills affected by amoebic gill disease. (B) Gill lesion from an amoebic gill disease-affected fish (A) showing epithelial hyperplasia, lamellar fusion and amoebae, containing one or more endosymbiotic *Perkinsella amoeba*-like organisms (arrow), associated with pathological changes to the gill epithelium (arrowhead). Haematoxylin-eosin staining. Scale bar 50 μm.
2.3. Histology and in situ hybridisation (ISH)

Gill arches from moribund fish with typical signs of AGD (gross lesions as described by Adams et al., 2004) were fixed in 10% phosphate-buffered formalin for at least 24 h, dehydrated through a graded alcohol series and processed for histological examination. Sections (5 μm) of the gill arch were stained with haematoxylin and eosin (H&E) and observations were made under a light microscope (Leica DM1000, Hamburg, Germany). The prevalence of AGD was determined by calculating the percentage of fish which were AGD positive on the basis of histology. AGD positive fish were defined as those which had amoebae containing PLOs in close association with hyperplastic epithelial-like cells in histological sections of gills.

For in situ hybridization (ISH), serial sections (7 μm) were placed onto Polysine glass slides (Menzel-GLäser, Braunschweig, Germany) and dried overnight at 37 °C. Sections were hybridized with one of three digoxigenin (DIG)-labeled 18S rRNA oligonucleotide probes specific for closely related Neoparamoeba species, N. perurans, N. pemaquidensis and N. branchiphila as previously described (Young et al., 2007). Positive and negative (no probe) controls were run in parallel with each ISH experiment by hybridizing each probe with a section containing representative isolates of each Neoparamoeba species termed an “amoebae array” as previously described (Young et al., 2007). Tissue sections were incubated for up to 1 h with premixed BCIP/NBT solution (Sigma-Aldrich, Castle Hill, New South Wales, Australia) for color development.

2.4. PCR of N. perurans DNA from salmon clinical samples

A 636 bp fragment of the N. perurans 18S rRNA gene was amplified from 20 ng of genomic DNA isolated from the gills of Atlantic salmon presumptively diagnosed with AGD as previously described (Young et al., 2007).
et al., 2008b). PCR reactions containing either no template or plasmid DNA housing the full-length 18S rRNA gene of *N. perurans* (EF216901) were used as negative and positive controls, respectively. PCR reactions were electrophoresed through 2% agarose/tris-borate EDTA buffer and visualized by staining with ethidium bromide.

2.5. PCR product amplification and sequencing

Universal oligonucleotide primers complementary to conserved regions of the eukaryotic 18S rRNA gene were used to PCR amplify and sequence the entire *N. perurans* 18S rRNA gene from genomic DNA isolated from AGD-affected Atlantic salmon gill tissues collected in this study. Briefly, genomic DNA was extracted from gill tissues using a Tissue DNA Kit (Omega-Biotek, USA) as per the manufacturer’s instructions. All amplification was performed as previously described (Dyková et al., 2005), except annealing temperatures were raised to 60°C and extension times were reduced to 30 s. PCR products were purified with EZNAP® Extraction Gel Kit (Omega-biotek, USA) as per manufacturer’s instructions. The 18S gene sequence from this study was deposited in GenBank (National Center for Biotechnology Information, US National Library of Medicine, Bethesda, MD) with the Accession Number GQ407108.

2.6. Neoparamoeba phylogeny using the 18S rRNA gene

The Chilean 18S rRNA gene sequence was assembled using ContigExpress (Vector NTI Advance v11, Invitrogen, USA) and compared with 18S rRNA gene sequences from 46 isolates of *Neoparamoeba* and an outgroup consisting of *Korotneveella hemistylolepis* (AY121850), *Korotneveella stella* (AY686573, AY183893) and *Pseudoparamoeba pagel* (AY686576, AY183891) obtained from GenBank. All 18S rRNA gene sequences were aligned using AlignX Software (Vector NTI Advance v11, Invitrogen, USA) (gap opening/gap extension penalty = 8/2) and exported to NEXUS format using BioEdit (Hall, 1999). Genetic distances among 18S rRNA gene sequences were calculated as mean character differences using PAUP*+ Version 4.0b10 (Swofford, 2002). Maximum likelihood (ML) analysis was conducted on the described aligned dataset using PAUP* Version 4.0b10 (Swofford, 2002) incorporating the GTR+G+I model selected using Akaike information criteria (AIC) in Modeltest 3.7 (Posada and Crandall, 1998). Maximum likelihood analysis bootstrap support (1,000 replicates) for each tree node was obtained using PHYML (Guindon and Gascuel, 2003) with the GTR+G+I model. The consensus tree was visualised using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (Tamura et al., 2007).

2.7. Statistical analyses

The relationship between monthly percentage mortality and *Caligus rogercresseyi* intensity, salinity and temperature were assessed separately for each farm using Pearson’s correlation, all data were analysed using SPSS 16.0 statistical software (SPSS Inc, USA). A *P*-value < 0.05 was considered a significant difference for all cases.

3. Results

3.1. Epidemiology

The highest monthly mortality on Farm 1 coincided with highest temperature and highest salinity (Tables 1 and 2). However, overall mortality patterns were inconsistent between farms (Table 2). The fish were of poor condition, displaying signs of lethargy, respiratory distress and surface swimming. Food intake was low leading to the reduction in growth rate (up to 25%) and increased feed conversion rate (information provided by the salmon farms, data not shown). On the basis of gross gill lesions AGD prevalence was estimated as 40–60%. Concurrently, the fish were affected by a heavy *C. rogercresseyi* infection (prevalence 100% at each sampling time and farm, maximum mean intensity 34), and subsequent to their removal as mortalities, a new recruitment of non-performing fish (runs) was observed. However, the monthly mortality rates were not directly related to the mean intensity of *C. rogercresseyi* infection except for Farm 1, where both the highest monthly mortality rate and highest mean intensity of *C. rogercresseyi* infection occurred within the month of November 2007 (*r* = 0.77, *P* = 0.04, Table 2). On Farm 2 the highest mortality was recorded in May but the highest mean intensity of *C. rogercresseyi* infection was in November and there was no significant relationship with monthly mortality (*r* = 0.542, *P* = 0.209, Table 2). Similarly, there was no relationship on Farm 3, where the highest mortality was in October but the peak of *C. rogercresseyi* infection was in July (*r* = −0.108, *P* = 0.818, Table 2). There was a significant positive correlation between monthly mortalities and temperature on Farm 2 (*r* = 0.852, *P* = 0.015), however there was no relationship between mortality and water temperature on the other two farms. The cumulative mortalities from May to November 2007 were 11.38% on Farm 3 and 37.85% on Farm 2, and it reached 53.82% at Farm 1 leading to grading and removal of the non-performing fish after the last sampling in November 2007.

Alphamax (PHARMAQ) became available only in September 2007 and has been used since then by Chilean salmon industry. Its use on Farm 1 did not result in the decline in the intensity of infection. The treatment with Alphamax in August 2007 on the other two farms seemed to cause a decrease in the infection intensity in September, however the intensity increased again in October and reached its peak in November so there was no longer term effect.

The gills of affected fish were pale and multifocal, diffuse white AGD-like lesions were observed (Fig. 2A). *Piscirickettsia salmonis*, IFNV or *Vibrio ordalli* were detected in any of the fish sampled and all the farms remained negative for ISAV until 2008 (Sernapesca, National Fisheries Services, Chile).
3.2. Environmental conditions

During the outbreak the salinity ranged from 27.9‰ in August to 32.8‰ in November and water temperature ranged from 9.0 °C in August to 11.9 °C in November (Table 1). The rainfall was lower than the fifteen year average, in particular in May 2007 it was only 36.2 mm, while May average for 1993–2007 was 206.4 mm (Table 1).

3.3. Histology

Changes in the gills were characterized by hyperplasia of epithelial-like cells across the gill filaments. This resulted in the fusion of lamellae and the development of round to oval interlamellar vesicles. Multiple rows of attached amoebae, each containing at least one PLO were associated with the affected tissue (Fig. 2B). Additionally, hyperplasia of mucous cells was observed in the gills. The severe and diffuse hyperplasia of epithelial-like cells with the presence of amoebae containing PLO and the development of interlamellar vesicles is recognized as characteristic for AGD (Fig. 2B). The overall prevalence of AGD defined as the presence of amoebae in histological sections was 67.9% for Farm 1, 66.7% for Farm 2 and 63.4% for Farm 3. While the lesion severity was not quantified (for example as percentage of filaments affected), the lesions were severe and characteristic of untreated AGD outbreak with most of filaments affected by extensive hyperplasia (Figs. 2 and 3).

3.4. In situ hybridisation

The N. perurans-specific probe hybridized with all trophozoites in AGD-affected Atlantic salmon gill sections prepared from three replicate fish (Fig. 3D–E). In serially-sectioned gills, neither the N. pemaeoidensis nor the N. branchiphila-specific probes hybridised with any trophozoites (Fig. 3F–L). Neoparamoeba species-specific probes hybridised with the corresponding Neoparamoeba species on the amoebae array while no signal was detected in trophozoites on the amoebae array when the probes were omitted from the hybridisation procedure. This occurred in all hybridisation experiments (results not shown).

3.5. Diagnostic PCR

Employing the N. perurans-specific diagnostic PCR, similar sized PCR amplicons were generated from all gill tissue samples from farms 1, 2 and 3 of the expected size (636 bp) (Fig. 4).

3.6. Phylogenetic analysis of Neoparamoeba 18S rRNA gene sequences

The 18S rRNA gene amplified from amoebae isolates derived from the gills of AGD-affected Atlantic salmon in Chile clustered with N. perurans isolates globally with strong bootstrap support (Fig. 5). The Chilean 18S rRNA gene sequence (GQ407108) has 98.4–99.2 similarity with N. perurans sequences from Australia and 99.6% similarity with N. perurans from Norway (EU326494) (Table 3).

4. Discussion

This is the first confirmed record of an AGD outbreak in Atlantic salmon farmed in Chile, caused by Neoparamoeba perurans. Previous records from Chile were incidental findings of amoebae on the gills of
fish suffering from other epizootics (Nowak et al., 2002) and an immuno-fluorescence antibody test (IFAT) previously used to confirm the identity of the agent (Nowak et al., 2002) was shown to lack species specificity (Morrison et al., 2004). Our study has linked the characteristic AGD gill histopathology with the presence of N. perurans trophozoites in close association with the AGD-affected gills from Chilean farmed Atlantic salmon. This extends the known geographical range of N. perurans and is consistent with previous findings that N. perurans is the only known aetiologic agent of AGD.

The most likely reason for the timing of this AGD epizootic was the significantly lower rainfall over the year, particularly in May and July, leading to abnormally high salinity. High salinity is considered to be the most important environmental risk factor for AGD (Clark and Nowak, 1999; Munday et al., 2001). Indeed, low rainfall has been associated with epizootics of AGD in Tasmania (Clark and Nowak, 1999) and Ireland (Rodger and McArdle, 1996). The AGD epizootics reported from Norway and Scotland appeared to be associated with higher than average water temperature (Steinum et al., 2008). While in the AGD outbreak described here the temperature was reasonably low, ranging from 9 to 12 °C, AGD epizootics at similar temperature ranges have been reported in salmonids farmed in the USA (Kent et al., 1988; Douglas-Helders et al., 2001).

Caligus rogercresseyi is the dominant copepod species affecting farmed salmonids in Chile (González and Carvajal, 2003). The Caligus spp. infections in Atlantic salmon farmed in Chile have been controlled at a level of 5 copepods/fish until 2004 (Zagmutt-Vergara et al., 2005). However, since then, the mean intensity of infection reached 34 parasites while the prevalence was 97% (Rozas and Asencio, 2007). This, however, since then, the mean intensity of infection reached 34 parasites while the prevalence was 97% (Rozas and Asencio, 2007). This extends the known geographical range of Neoparamoeba perurans and is consistent with previous findings that N. perurans is the only known aetiologic agent of AGD. However, since then, the mean intensity of infection reached 34 parasites while the prevalence was 97% (Rozas and Asencio, 2007). This extends the known geographical range of Neoparamoeba perurans and is consistent with previous findings that N. perurans is the only known aetiologic agent of AGD.

Together, these data suggest that N. perurans is a causal agent of AGD in Chile. However, the exceptionally high level of co-infection with C. rogercresseyi may have contributed to the observed epizootic. A more sensitive technique for isolating amoebic gill disease virus from asymptomatic carrier rainbow trout, Salmo gairdneri Richardson, Journal of Fish Diseases 5, 285–292. Bush, S., Sevadal, S., Horsberg, T., 2008. Sensitivity assessment of Caligus rogercresseyi to emamectin benzoate in Chile. Aquaculture 282, 7–12.

Table 3

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<tr>
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<th>N. perurans (N)</th>
<th>N. perurans (Ch)</th>
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Acknowledgements

This work formed part of a project of ADL Diagnostic Chile Ltda. and received funds from Chilean Economic Development Agency (CORFO). We thank Dr David Croman (UPEI, Canada) and Dr Jeremy Carson (Department of Primary Industries, Parks, Water & Environment, Launceston, Australia) for their valuable contribution to the diagnosis of AGD in Chile. We would like to thank Ms Victoria Valdenegro for doing the statistical analyses. The monitoring of copepods was a part of Chilean salmon industry surveillance program coordinated by INTESAL (Instituto Tecnológico del Salmón). The monitoring of ISA followed “ISA Contingency Plan” developed by Sernapesca (National Fisheries Service) in August 2007 (Resolution Number 1670) and then included in the Sanitary Specific Program to ISA Surveillance and Control.

References


Table 3

Percent similarity among aligned 18S rRNA gene sequences of Neoparamoeba used for phylogenetic analysis calculated by pairwise alignment. The outgroup consists of sequences representative of Korotnevetella hemistylepis (AY121850), Korotnevetella stella (AY686573, AY183893) and Pseudoparamoeba pagi (AY868576, AY183891). Isolates of Neoparamoeba perurans from Tasmania (T), Norway (N) and Chile (Ch) were examined individually. The sequence length for which these sequence similarities were obtained was 2079 bp.

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<td>N. perurans (Ch)</td>
<td>100.00</td>
<td>96.63–100.00</td>
<td>95.22–95.10</td>
<td>93.88–98.78</td>
<td>95.82–98.60</td>
</tr>
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References
