Development of PCR assay for detection of *Neoparamoeba perurans* and comparison of histological diagnosis

M. Rozas*, H. Bohle, R. Ildefonso and P. Bustos

*ADL Diagnostic Chile Ltd, Diagnostic and Biotechnology Laboratory, Puerto Montt, Chile*

**Abstract**

The recent description of Amoebic Gill Disease (AGD) and *Neoparamoeba perurans* in Atlantic salmon (*Salmo salar*) farmed in Chile has necessitated the development of more reliable and sensitive diagnostic tests. Final diagnosis of infection is normally confirmed by histology. However, the correlation between gross gill lesions and histological lesions is generally unclear. In the current study, moderate concordance level (k=0.5319) between gross pathology and histology was observed. The sensitivity and specificity of gross pathology was 77.91% and 71.05%, respectively. *Neoparamoeba* spp. are considered morphologically indistinguishable therefore by using histopathology limits the capacity to characterise the causative agent and it can be time consuming. We developed a PCR assay to amplify the *N. perurans* 18S rRNA gene from gill clinical samples of AGD-affected fish. High concordance level (k=0.95) between PCR and histological examination was observed. The sensitivity and specificity of PCR assay was 94.64% and 97.06%, respectively. The PCR-based assay provides a rapid tool that will be useful to the diagnostic routine for AGD in Chile.

**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* (Young et al., 2007) and has been reported from Australia, Ireland, Norway, USA, Scotland, Spain, Japan and Chile (Munday, 1986; Rodger and McArdle, 1996; Young et al., 2008a; Steinum et al., 2008; Bustos et al., 2011). AGD is grossly characterised by the presence of multifocal lesions that appear as pale gill tissue (Munday, 1986). The presence of these gross lesions allows presumptive diagnoses of AGD but histological examination of gill tissues has been a reliable method for diagnosing AGD (Dyková et al., 2000).

The final diagnosis is confirmed when trophozoites that possess one or more endosymbiotic *Perkinsela amoebae*-like organisms (PLOs) (Dyková et al., 2003) are detected in close association with hyperplastic gill lesions (Dyková et al., 2000). Whether or not a gross gill lesion is indicative of a histological lesion is largely unclear and both methods only diagnose the problem after an outbreak has occurred (Clark and Nowak, 1999). *Neoparamoeba* spp. are however considered morphologically indistinguishable (Dyková et al., 2000) therefore characterisation and identification of the infectious agents to species using histology only may be limited leading to presumptive identification of the infectious agent only (Young et al., 2008b).

* Corresponding author's e-mail: mrozas@adldiagnostic.cl
PCR-based assays provide advantages over other methods used for the routine diagnosis of parasitic infection particularly differentiation is required between morphologically identical species (Weiss, 1995). The emergence of *N. perurans* as aetiological agent of AGD in Chile (Bustos et al., 2011) led us to develop a PCR assay to detect *N. perurans* in clinical samples from AGD-affected Atlantic salmon and the ability to diagnose AGD was evaluated by comparing the sensitivity and specificity of the diagnostic PCR assay with lesions observed from histological examination of gill tissue.

**Materials and Methods**

**Sampling and histological processing**

Gill samples were collected from 90 fish from 8 Atlantic salmon seawater farms that were affected with AGD and 34 fish from 4 farms that were not experiencing cases of AGD. All fish were necropsied and the clinical signs and gross pathology were noted. Gill arches were fixed in 10% phosphate-buffered formalin for at least 24 h dehydrated through a graded alcohol series and 5 μm sections were stained with haematoxylin and eosin (H&E) and observed microscopically. AGD positive fish were defined as those which had amoebae containing PLOs in close association with hyperplastic epithelial-like cells.

**Development of PCR primers for N. perurans**

Full-length sequence of *N. perurans* 18S rRNA gene obtained from genomic DNA isolated from AGD-affected Atlantic salmon gill tissues was used (GQ407108) and 18S rRNA sequences consensus were obtained from GenBank as previously described (Bustos et al., 2011). *Neoparamoeba* spp. 18S rRNA gene full sequences alignment were performed by AlignX (Vector NTi Advance v11, Invitrogen, USA) and checked with BioEdit (Hall, 1999). We synthesised 2 oligonucleotides, forward primer (Np18sF1) 5'-CTT ACT AGA CTT TCA TTA CAC-3' were respectively paired with the reverse primer (Np18sR2) 5'-TCT AAG CAG AAC GAA CTT TC-3' and tested for amplification of *N. perurans* 18S rRNA gene.

**PCR of N. perurans from salmon farm samples**

Genomic DNA from gill tissue was extracted using Tissue DNA Kit (Omega-Biotek, USA) as per the manufacturer’s instructions. Atlantic salmon genomic DNA was used as host control while samples without template and plasmid DNA housing the full-length 18S rRNA of *N. perurans* gene (GenBank EF216901), were used as negative and positive control, respectively. All amplification was performed on T3 thermocycler (Biometra, UK) in final volume of 25 μL containing 0.625 μL polimerase Platinum® Taq (Invitrogen, USA), 250 μM of each triphosphate deoxinucleotides, 20 ng DNA template, 1.5 mM MgCl₂, and 0.4 μM of each oligonucleotide. Cycling conditions were 94°C for 3 min; 94°C for 30 sec, 50°C for 30 sec, and 72°C for 45 sec for 35 cycles; and 72°C for 10 min. PCR reactions were electrophoresed through 2% agarose/tris-borate EDTA buffer and visualized by staining with ethidium bromide.

**Sequence verification of PCR products**

Of all the gill DNA samples that had a positive band with the N. perurans primers, 4 were randomly chosen for sequencing. PCR products were purified with EZNA® Extraction Gel Kit (Omega-Biotek, USA) as per the manufacturer’s instructions and sequenced using the ABI PRISM 310 DNA analyzer (Applied
Biosystem, USA). DNA sequences were aligned and compared to Neoparamoeba spp 18S rRNA gene sequences.

**Statistical analysis**
The sensitivity, specificity, negative and positive predictive values were calculated by designating the histological examination as a gold standard. The data were analysed using the chi-square test of independence to compare the results obtained by the different diagnostic tests and kappa value (k) was also computed (Cichetti and Feinstein, 1990). Computations were performed using Software STATGRAPHICS Plus 5.1 (2002).

**Results**
**Gross pathology and histopathology lesions**
Gross pathology was characterised for pale gill and raised, multifocal white mucoid patches on the external surface gills in the 71.43% (95/124) of fish analysed (Figure 1). In addition, 42.11% (56/124) of fish showed moderate abundance of *Caligus rogercresseyi*. Histological observations revealed AGD lesions with variable hyperplasia, lamellar fusion, presence of interlamellar vesicles and multiples amoebae adhered on gill epithelium (Figure 2). Amoebae were often seen adhered to or in close proximity to lesions and sometimes entrapped within interlamellar vesicles.

In total 91.13% (113/124) of fish presented different hyperplastic degrees on gills lamellae whilst 63.70% (79/124) showed a variable numbers of both uniform and pleomorphic amoebae with basophilic nucli and eosinophilic parasomes represented by one or more endosymbiotic perinuclear eosinophilic bodies (PLOs). Interlamellar vesicles were noted in 54.03% (67/124)

![Figure 1. Gross pathology of Atlantic salmon gills affected by AGD. Multifocal white patches on dorsal surface of gills.](image-url)
whilst 52.42% (65/124) showed fusion of secondary lamellae, respectively. The severity of histological lesions did not always correspond with the presence of amoebae on gills tissues and occasionally the structural lesions without amoebae were observed.

**Application of N. perurans PCR to field-based samples**

The oligonucleotides designed and used for detection of *N. perurans* showed amplicons of expected size (462 pb) from all analysed fish (Figure 3). PCR products were obtained from *N. perurans* genomic DNA from gill tissue but it were not observed with DNA templates from others non related *Neoparamoeba* spp and Atlantic salmon. The DNA sequences obtained from gill samples showed > 99% homology with *N. perurans* 18S rRNA gene (data not showed).

**Sensitivity, specificity and association analysis**

The sensitivity of gross pathology was 77.91% (67/86) and the specificity was 71.05% (11/38) (Table 1). The positive and negative predictive value was 86 and 59%, respectively. There was a moderate concordance level (k=0.53) between gross pathology and histological examination. The sensitivity of PCR assay was 94.64% (53/56) and the specificity was 97.06% (66/68) (Table 2). The positive and negative predictive value was 96% in both cases. High concordance level (k=0.95) between histological examination and PCR was observed. The 66.67% (8/12) of analysed farms by histology and PCR were AGD positives but the AGD prevalence into each farm was observed between 23.41 and 100%. The 45.16% (56/124) and 44.35% (55/124) of fish were AGD positives by histology and PCR, respectively.

![Figure 2. Gill lesion from an AGD-affected fish (H&E). Epithelial gill hyperplasia with an attached amoebae (black arrow), secondary lamellae fusion (white asterisk) and presence of interlamellar vesicles (black asterisk) (bar 80 μm). Magnification showing amoebae details of nucleus with an amphiphilic core surrounded by an irregular basophilic ring (black arrow) and parasomes appeared with eosinophilic cytoplasm (black head arrow) (bar 20 μm).](image)
Figure 3. Polyacrylamide gel of PCR products amplified from four clinical samples. Lane M, molecular weight standard (100 pb), Lane 1, Extraction negative control; Lane 2 and 9, PCR negative control; Lane 3, Negative clinical sample; Lane 4 - 7, Positives clinical samples; Lane 8; *N. perurans* DNA plasmid (EF216901), Positive control.

<table>
<thead>
<tr>
<th>Table 1. Contingency table with numbers of fish diagnosed as AGD positives or negatives by gross pathology and histology.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gross pathology</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Positive</td>
</tr>
<tr>
<td>Negative</td>
</tr>
<tr>
<td><strong>Total</strong></td>
</tr>
</tbody>
</table>

$X^2=33.34$, df=1, $p<0.000$ ($p<0.01$ with 99% confidence), $k=0.5319$

<table>
<thead>
<tr>
<th>Table 2. Contingency table with numbers of fish diagnosed as AGD positives or negatives by PCR and histology.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PCR</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Positive</td>
</tr>
<tr>
<td>Negative</td>
</tr>
<tr>
<td><strong>Total</strong></td>
</tr>
</tbody>
</table>

$X^2=104.63$, df=1, $p<0.000$ ($p<0.01$ with 99% confidence), $k=0.95$
Discussion
The current study of AGD-affected Atlantic salmon in Chile has shown that both gross pathology and histological lesions are identical to AGD previously described (Munday et al., 1986; Bustos et al., 2011). Lesion numbers and severity were significantly higher in the dorsal region in this study which coincides with what was previously described (Adams and Nowak, 2001) but contrasts with some of the evidence that indicates a mainly ventral occurrence of gross lesions on the field (Clark and Nowak, 1999). Water flow through the dorsal region of the gills is quite possibly retarded because of the influence of the cranial lobes, permitting increased contact time between host and pathogen (Adams et al., 2004).

Clark & Nowak (1999) described a lack of correlation for light infections when investigating a consensus between gross lesion scores and lesions presenting histologically. Disparity between gross and histopathological findings was mainly attributed to sampling technique during gill excision therefore removal of grossly affected tissue and subsequent histological examination will improve diagnostic accuracy (Adams et al., 2004). The histological lesions of AGD occasionally were observed without amoebae in accordance to what was previously described (Nowak and Munday, 1994; Adams et al., 2004).

The histology is able to diagnose AGD but is unable to identify the type of agent because all members of Neoparamoeba genus are considered morphologically indistinguishable (Dykova et al., 2000; Young et al., 2007). Before N. perurans was confirmed as the only causative agent of AGD in marine fish around the world (Young et al., 2007; Young et al., 2008a).

The PCR developed in this study was able to detect N. perurans genetic material in gills of fish with gross pathology and histological lesion characteristic of AGD. The sensitivity and specificity of the PCR assay in comparison with histology were all > 90%, therefore the PCR assay would be useful to detect AGD. The specificity was higher than the sensitivity, which indicates that the test was more reliable at identifying the fish that are negative, that are unaffected, than for identifying as positive those fish that are affected. Therefore, the PCR that was developed is a simple assay with advantages of the sensible test and can determine the presence or absence of N. perurans in all stages of its life cycle and in early infection phases. PCR assay would be a test with high potential that can be applied in the AGD routine laboratory diagnosis. In addition, the severe impacts of AGD on Chilean Atlantic salmon industry have led to an increasing interest in assessing the epidemiology of N. perurans in the associated marine environment, with the purpose of developing better farm management strategies for controlling the disease. The test is useful specifically for AGD and use of histology would allow for assessment of the severity of infection and ensure that any other conditions could be assessed and recorded.

Acknowledgments
This work formed part of a project of ADL Diagnostic Chile Ltd. and was financed from Chilean Economic Development Agency (CORFO).

References
Adams MB and Nowak BF (2001). Distribution and structure of lesions in the gills of Atlantic salmon, Salmo salar L., affected


