**Generation of *Paramoeba perurans* clonal cultures using flow cytometry and confirmation of virulence.**

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**Abstract**

Amoebic gill disease (AGD) in farmed Atlantic salmon is caused by the amoeba *Paramoeba perurans*. The recent establishment of *in vitro* culture techniques for *P. perurans* has provided a valuable tool for studying the parasite in detail. In the current study, flow cytometry was used to generate clonal cultures from single sorted amoeba, and these were used to successfully establish AGD in experimental Atlantic salmon. The clonal cultures displayed differences in virulence, based on gill scores. The *P. perurans* load on gills, determined by qPCR analysis, showed a positive relationship with gill score, and with clonal virulence, indicating that ability of amoebae to proliferate and/or remain attached on gills may play a role in virulence. Gill scores based on gross signs and histopathological analysis were in agreement. No association between level of gill score and specific gill arch was observed. It was found that for fish with lower gill scores based on histopathological examination, gross examination andqPCR analysis of gills from the same fish were less successful in detecting lesions and amoebae respectively.

Key words: *Paramoeba, perurans*, flowcytometry, clonal, culture, virulence

**Introduction**

Amoebic gill disease (AGD) is a term used to describe a gill induced disease, caused by amoebae, in farmed marine fish species including Atlantic (*Salmo salar* L.), Chinook (*Oncorhynchus tshawytscha* (Walbaum)) and Coho (*Oncorhynchus kisutch* (Walbaum)) salmon, rainbow trout (*Oncorhynchus mykiss* (Walbaum)), turbot (*Scophthalmus maximus* (L.)), sea bass (*Dicentrarchus labrax* (L.)), sea bream (*Diplodus puntazzo* (Walbaum))*,* Ayu (*P*lecoglossus altivelis (Temminck & Schlegel)), blue warehou (*Seriolella brama* (Günther)) *,* Ballan wrasse (*Labrus bergylta* (Ascanius)),corkwing wrasse (*Symphodus melops* (L.)) and Lumpfish (*Cyclopterus lumpus* L.) (Munday *et al.,* 1993; Kent *et al.,* 1988; Dykova *et al.,* 1998; Dykova & Novoa2001; Munday *et al*., 2001; Adams *et al.* 2008; Steinum *et al*., 2008; Crosbie et al. 2010; Karlsbakk et al. 2013, Haugland et al. 2016). Amoebic gill disease has caused significant issues for salmon farming in a number of countries, in particular, with respect to persistent occurrence, in Tasmania, and recently in Northern Europe including Ireland, Scotland and Norway (Munday et al., 2001; Nowak, 2012; The fish site: October 2014Sustainable Aquaculture Digital*;* VKM, 2014; Shinn et al, 2015;). The disease in salmonids is characterised by raised lesions on gills due to lamellae epithelial hyperplasia, hypertrophy, excessive mucus (diffuse or in patches), oedema of the epithelium, cell atrophy and reduction in chloride cell numbers (Munday *et al*., 1990; Adams & Nowak, 2001, 2003; Peyghan & Powell, 2006).

With regards to Atlantic salmon, and a number of other species, the amoeba *Paramoeba perurans*, a member of the Sarcomastigophora (Paramoebidae) family has been identified or confirmed as the causative agent of AGD (Crosbie *et al*., 2012). *Paramoeba* spp. are considered free living in general, and ubiquitous in the temperate marine environment (Page 1970, Jones, 1985, Dyková *et al*.. 2005, Kudryavtsev *et al*., 2011, Feehan *et al*., 2013) but is facultatively parasitic, giving rise to disease when infesting fish, at least in aquaculture situations. Previously, AGD in salmon was attributed to the closely related species *Neoparamoeba pemaquidensis* (Kent *et al*., 1988; Munday *et al*., 2001; Wong *et al*., 2004), but researchers were unable to induce AGD in experimental environments with 34 and 98 day old verified *N. pemaquidensis* cultures (Morrison *et al*., 2005). Subsequently *P. perurans* was identified in the amoebae populations from AGD infested salmon gills and shown to fulfil Koch’s postulates when transferred to naïve fish (Crosbie *et al*., 2012). In 2012 a method for culturing *P. perurans* *in vitro* was established (Crosbie *et al*., 2012) and this has facilitated study of the parasite under controlled conditions. Previously, longer term amoebae cultures (where presence of *N. pemaquidensis* was confirmed in culture) isolated from infected salmon gills failed to cause disease in aquarium challenges, as opposed to amoebae isolated only 72 h prior to challenge (Morrison *et al*., 2005). Assuming that *P. peruans* had been present initially in cultures, then loss of virulence has been considered in some instances to their being outcompeted by *N. pemaquidensis* or other organisms co-isolated in initial gill preparations. It has been questioned whether the cultures, used in the study of *P. perurans* and AGD, represent pure *P. perurans*. Isolation of clonal cultures, though reducing genetic variability, would help assure that data on biological, infective, or other parameters generated using culture material attributed to *P. perurans,* were robust. Dilution to single cell level is the traditional approach to generating clonal cultures. However, it can sometimes be difficult to ascertain the presence of only a single cell giving rise to the culture, and it can be time consuming if many single cells are required to be isolated. In response to this a flow cytometric approach was tested for isolating single cells from which cultures of *P. perurans* were established. A polyclonal *P. perurans* culture was established at Marine Scotland Science (MSS) in 2012 from infested Atlantic salmon farmed on the west coast of Scotland and used as material to test this approach. The resulting monoclonal *P. perurans* cultures were then used in a challenge to determine if theywere as effective as crudely isolated *P. perurans* polyclonal cultures in inducing amoebic gill disease in Atlantic salmon.

**MATERIALS AND METHODS**

***Paramoeba perurans* *in vitro* culture**

An *in vitro* culture of *Paramoeba perurans* was established at this laboratory in November 2012 from gills of infested farmed Atlantic salmon from the west coast of Scotland. Cultures were isolated as described in Morrison *et al*., (2004), Crosbie *et al*. (2012), with some differences. A number of individual gill arches were received in sea water at ambient temperature from the farm source. The gill samples were transferred into 35 salinity, 0.22 μm filtered (Steritop™ 0.22 μm polyethersulfone (PES) membrane filters, Merck Millipore, Fisher Scientific), sterile sea water (SSW), in 50 mL tubes and shaken vigorously for 30 s to detach amoebae. The amoebae were allowed to settle for up to four hours at room temperature in T-25 flasks (Greiner). Material in suspension was poured off, and attached amoebae subsequently detached from flask base following incubation for 30 mins at room temperature in 0.5 mL of trypsin 2.5% (Gibco), with intermittent tapping of flasks. The detached amoebae were centrifuged at 400 x g for 10 min and washed once in SSW containing 5 U/mL penicillin and 5 µg/mL streptomycin (Life Technologies). The amoebae were resuspended in sea water containing antibiotics as previously, and added to malt yeast agar (MYA; 0.01 % (w/v) malt extract, 0.01 % (w/v) yeast extract, 2 % (w/v) bacteriological agar, (Oxoid Ltd., UK in SSW) plates with SSW containing antibiotics, as previously, to a final volume of approximately 7 mL. The amoebae were placed at 15°C and monitored daily for propagation. Amoebae in suspension were subsequently subcultured and grown on fresh MYA plates in SSW but without antibiotics. The antibiotic wash reduced but did not eliminate bacteria during establishment of the amoeba culture. Therefore the culture was xenic, containing contaminating microflora co-isolated with amoebae from fish gills which, persisted despite the initial antibiotic treatments. Presence of *P. perurans* was confirmed by 18S rDNA species specific qPCR (Fringuelli *et al*., 2012), and traditional PCR (Young *et al*., 2008). This culture is subsequently referred to as the “polyclonal” *P. perurans* culture, i.e. established from the population of amoebae on gills rather than individual amoebae.

**Isolation of *Paramoeba perurans* clonal cultures using flow cytometry**

Amoebae from the polyclonal culture (subcultured 5-8 times since initial isolation) were concentrated by centrifugation at 400 x g for 10 min at room temperature. Approximately 200,000 cells were resuspended in 1 mL of SSW. The amoebae preparation contained a mixture of amoeba, single celled yeast-like organisms and bacteria. Ciliate contaminants were not observed. Although *P. perurans* presence was confirmed by qPCR and PCR in the polyclonal culture, other amoeba species may also have been present at low numbers. Samples were processed using flow cytometry at the Iain Fraser Cytometry Centre, Institute of Medical Sciences, University of Aberdeen. The samples (amoebae and other culture constituents in SSW) were run initially on a 5 laser BD LSR Fortessa™ cell analyser (BD BioSciences) and the data subsequently analysed using DiVa and FlowJo software software, to obtain information on relative size and autofluorescenec of hetereogenous populations in the culture. To establish the size range of the different populations within the sample, and knowing the size of the amoebae to be variable but generally ≥15 µm (own observations, Karlsbakk *et al*., 2013), 15 µm size calibration microspheres (Size Calibration beads kit Invitrogen F1383815 µm component F) were run along with samples. Populations were separated both on forward scatter (FSC, size) and side scatter (SSC, complexity), and autofluorescence. Particles ≥ 15µm were identified on FSC v SSC plots. Fluorescence intensities in multiple wavelength channels (in total 18) from 5 lasers: UV (355 nm), violet (405 nm), blue (488 nm), yellow-green (561 nm) and red (640 nm), were compared to determine the optimum autofluorescence emissions. Autofluoresence was observed optimally in the violet (405 nm) and UV (355 nm) excited channels. Having identified the putative amoeba population within the heterogeneous sample on LSR Fortessa, the samples were then sorted on a BD Influx cell sorter. Populations of interest were identified and sorted using FSC (size) and autofluorescence from the UV laser (at emission wavelengths of 460 nm and 670 nm). Between 500 and 2000 cells from 5 different populations (P1-P5), differentiated based on scatter and/or autofluoresence, were sorted into individual wells of a 24-well plate (Greiner) for examination under light microscopy. Single cells from the two largest populations based on particle size were sorted into individual wells on 96-well plates (Greiner GMH), containing 200 μl of 0.22 μm SSW overlying a MYA in sea water layer, using the single sort mode of the instrument.

Wells were examined for presence of single amoebae under light microscopy, and monitored intermittently for 4 weeks. MYA underlays where clonal cultures developed were removed from wells in 96-well plates using a fine spatula with bent head and placed with amoebae (attached) facing downwards in 24-well plates (Greiner GMH), followed by subsequent removal of propagated amoebae suspended in overlay to 50 mm deep well Pertri dishes (Greiner GMH) with approximately 7 mL sterile sea water. Clonal cultures were monitored for continued propagation and initially subcultured every week, and then every two to three weeks as for polyclonal culture. Five clonal cultures were maintained.

The identity of the clonal cultures was confirmed as *P. perurans* using qPCR as previously described (Fringuelli *et al*., 2012) and traditional PCR (Young *et al*., 2008). The PCR products were purified using the QIAquick gel extraction kit (Qiagen) and sequenced directly, using the same primers as for PCR. Sequencing was performed by DNA Sequencing & Services (MRCPPU, College of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) on an Applied Biosystems model 3730 automated capillary DNA sequencer and using Applied Biosystems Big-Dye Ver 3.1 chemistry. Sequences were analysed using SequencherTM 3.0 software (Gene Codes Corporation, Ann Arbor, MI, USA). BLASTn searches in GenBank were used to confirm species identity.

**Experimental infection of Atlantic salmon with polyclonal and clonal cultures of *Paramoeba perurans***

*Preparation of Amoeba Cultures for the challenge experiment*

The polyclonal and clonal (B8, B9, CE6, CC3, C6) amoebae cultures were transferred to incubators set at 12°C 48 h before challenge to allow acclimatisation to the challenge temperature (12°C). The polyclonal and clonal cultures had been subcultured between 12-17 and 10-15 times respectively prior to use in the challenge. Amoebae in cultures were concentrated by centrifugation at 2200 x g for 10 min at 12°C, resuspended in 5 mL SSW, and counted using a dilution method as follows: a 50 µL aliquot of amoeba culture was added to 50 µL filtered sea water on a 96 well plate (Greiner GMH) in duplicate and three technical replicate 10 fold dilutions made from each initial dilution. The remaining amoebae were maintained in an incubator at 12°C and agitated at 150 rpm to reduce settlement onto the sides of tubes. The amoebae to be counted were allowed to settle in wells for 30 min. Total amoebae were counted at the dilution where amoebae in single figures were observed, and in the two higher dilutions. Amoebae concentration per mL of original suspension was calculated based on the average of all counts adjusted for dilution. Volumes sufficient to give 500 and 5000 amoebae L-1 in the final challenge volume were removed from the stock, diluted in SSW to a total volume of 40 mLs and placed on a shaking incubator (150 rpm) at 12°C until ready for transfer to the aquarium. While amoebae were being prepared for counting, viability was also tested. An aliquot of 100 µL resuspended amoebae was removed in triplicate and added to 0.35 μl of neutral red (NR) (Sigma–Aldrich, Germany), left for 30 min to allow NR uptake and then centrifuged at 2200 x g for 10 min. The supernatant was removed and amoebae resuspended in 100 μl of SSW and placed in 96-well plates. The amoebae were allowed to settle for 30 min before examining to facilitate counting. Viable amoebae were distinguished by uptake of NR stain.

*Preparation of the culture bacteria - negative control for the challenge experiment*

Bacterial species, originating from initial gill isolations, were maintained with amoebae in cultures during the various sub-culturing and clonal isolation procedures. To attempt to improve on the control for the experiment, and to determine if lesions might arise due to bacterial presence alone, the fish used as negative controls for the experiment were exposed to bacteria (without amoebae) from the cultures. Sea water overlays from the different clonal cultures and polyclonal culture were pooled and not tested as individual “negative” controls in the aquarium AGD challenge. The overlays were filtered twice through a Cyclopore™ Track Etched Membrane (GE Healthcare, Whatman, UK) of 3.0 μm pore size. The filtrate was checked under light microscopy (20X and 40X) for presence of amoebae.

*Exposure of fish to amoebae*

Atlantic salmon used in the experiment were held as pre-smolts and smolted at the Ellis aquarium, MSS, Aberdeen. Two weeks prior to challenge, the fish were transferred to tanks containing 350L 34-35 sea water with a flow through of 180 L h-1 and acclimatised to 12°C which was used throughout the experiment. Water supply to the aquarium is filtered twice, once through sediment with an approximate pore size of 40 µM, and then sand with an approximate pore size of 15 µM. The filtered water is then treated twice with UV at an initial exposure of 7571 J/m2 and a second exposure at 360 J/ m2. Five fish were screened for the presence of viral pathogens and *P. perurans* using qPCR, for bacterial pathogens using culture techniques, and gills were examined by histopathological analyses for presence of amoebae and signs of gill damage.

Fish were approximately 400g when challenged in groups of five (a stocking density of 5.7 kg m-3) with one of five clonal *P. perurans* cultures, or the polyclonal culture, and at 500 and 5000 amoebae L-1 (i.e. a total of 60 fish). An additional group of five fish comprised negative control and was exposed to amoebae culture media (filtered as described above) to remove amoebae but retain bacteria. The water flow was stopped and water level reduced to 120 L in each experimental tank. Aeration was maintained. Amoebae, at the appropriate concentrations, or filtered amoebae culture media, were added across the surface of the tanks (selected randomly) and water mixed using a net handle, specific to each tank. Five fish from the holding tanks were then added to each experimental tank and held for 4 hours in the 120 L standing water with aeration before water flow was recommenced at a rate of 180L h-1. Following challenge, until the end of the experiment, the fish were fed daily to 1% body weight using a mixture of the Skretting® Atlantic NA 500 + 50 PAX and LD PROTEC P200 + 60 PAX diets. Fish were terminally anaesthetised at three weeks post exposure using 0.1 g L-1 tricaine methanesulfonate **(**MS222) (Sigma).

*Quantitative PCR (qPCR) analysis of amoebae load*

A cotton swab was rubbed across the full upper surface of the uppermost gill on the right hand side of each fish (fish facing left) and transferred to 100% ethanol and stored for *P. peruans* qPCR analysis. The ethanol was removed by pipetting and replaced with ALT buffer (Qiagen) with 10 µL proteinase K (>600 mAU/mL, solution) and incubated at 56 ºC for at 3 hours. The buffer containing the swab was then vortexed and centrifuged for 3 min at 13000 rpm. DNA was extracted from supernatant using the MagAttract M48 DNA Mini kit (Qiagen) and the M48 Biorobot (Qiagen) according to the manufacturer’s instructions. DNA was eluted in 100 µL elution buffer (Qiagen). Quantitative PCR analysis to detect and quantify *P. perurans* in mucus samples was performed on a Lightcycler LC480 (Roche) using a TaqMan assay as described in Fringuelli *et al.* (2012). Reaction mixes contained 1 x Quanta Custom Toughmix(VWR), 900 nM each primer, 250 nM probe, 2 µL DNA in a total volume of 20 µL. Cycling conditions consisted of a preincubation step of 45°C and 95°C for 2 min each followed by 40 cycles of 95°C for 10 s, 60°C for 1min. Cp values above 35 for the Fringuelli *et al*. (2012) qPCR assay are considered unreliable during routine diagnostics at MSS. Salmon host elongation factor 1 alpha (ELFα) levels, as a proxy for swab sample size, were determined by qPCR following the same conditions as for the *P. perurans* qPCR assay, using 1 µL DNA in a total volume of 20 µL and primers as described in Bruno *et al* (2007). The *P. perurans* and host ELFα Cp values were converted to equivalent target amounts (ETAs) and the *P. perurans* ETA normalised to the amount of host ELFα ETA found in gill swab sample (as indicator of sample size).

*Histological analysis of gill score*

The operculum was raised using forceps to observe the cavity and branchial arches. The first gill arch from the left hand side of each fish (i.e. the fish faced left at necropsy) was excised and placed in 10% neutral buffered formalin (Cellpath) for a minimum of 24 h, routinely processed and paraffin wax embedded with dorsal side uppermost. Sections were cut at 3 µm and stained with haematoxylin and eosin (H&E). The sections were analyzed by light microscopy to identify lesion morphology and distribution patterns according to Adams & Nowak (2001). The number and size of lesions were recorded, along with the degree of pathological severity. The latter observations made up a gill score from 0 to 5 (none, light, mild, moderate, advanced, severe).

*Gross analysis of gill score*

All four gill arches were dissected from the right side of each fish and placed flat under sea water, dorsal surface uppermost, and photographed under standard lighting. For each fish a gross gill score from 0 to 5 was allocated for each gill arch based on the presence of raised, white spots and/or patches and proportion or extent of the combined lesions on its dorsal surface. Scoring for individual gill arches was adopted from the criteria used by Taylor *et al.* (2009) which scored across all gill arches to determine an overall gill score. The detail of treatment dose or group was unknown during gross and histological assessment.

*Statistical analysis*

Analyses were carried out using the R Statistical Environment (Ihaka & Gentleman, 1996) with the supplementary R packages ‘Hmisc’ version 3.16-0 and ‘ordinal’ version 2015.6-28. P values for inference tests are categorised as statistically significant when less than or equal to (≤) 0.05. Variation in gill-arch gross scores between culture, challenge dose and gill-arch were evaluated using an ordinal mixed effects regression model assuming a binomial error distribution and fitted by adaptive Gauss-Hermite quadrature approximation. Gill-arch gross scores (0,1,2,3,4 and 5) were modelled as a dependent function of the exploratory fixed effect factors of culture (B8, B9, C6, CC3, CE6 and polyclonal), dose (500 and 5000), gill-arch (1,2,3 and 4), two-way interactions between these, and the random effect of individual. The statistical significance of explanatory fixed effects, including posteriori tests of difference between specific cultures, was evaluated using likelihood ratio tests of nested stepwise-deletion models assuming a χ2 distribution. The association between gross and histology score (determined from first gill arch of right and left side of fish respectively) was estimated using the Gamma (G)-statistic (Goodman & Kruskal, 1954) and its difference from zero evaluated as described by Goodman and Kruskal (1963). The G-statistic was also used to estimate the association between the first gill-arch gross score and, where estimates were available, the quantity of amoeba DNA (in swab taken from the first gill arch) measured using qPCR.

The association between the gross score, as a dependent variable, and the proportion of individuals for which amoeba DNA was detected (based on converted and normalised amoebae ETA values), as an explanatory variable, was modelled using a Generalized Linear Model (McCullagh & Nelder, 1989) assuming a binomial error distribution and utilising a logit link function.

**RESULTS**

*Flow cytometric characterisation of populations in P. perurans polyclonal culture*

Five different populations within the sample were separated based on FSC and SSC, and on autofluorescence (Fig 1a, b). Following observation of the populations under light microscopy, two populations (P1 and P2) were observed to contain amoebae, with P2 comprised of smaller sized amoebae (with pseudopodia) and small rounded cells which may have been dead or stressed amoebae. No amoebae were observed in sorted cells from populations P3-P5. Autofluorescence was most defined for the amoebae using the UV laser in channels 460/50nm and 670/30nm, and facilitated differentiation from bacterial populations which showed low autofluorescence under these conditions.

*Generation of clonal cultures.*

Approximately 50% of the wells containing sorted single amoebae displayed propagation of amoebae after one week. Many of the amoebae following initial division after sorting had a rounded appearance, which may be indicative of high bacterial loads. Fig. 2 shows a clonal culture in the well of a 96-well plate, with round amoebae at centre, and more classical trophozoites at the periphery. Subsequent subculturing of the clonal cultures generated viable cultures with expected Paramoeba morphology.

*Confirmation of P. perurans species by qPCR, PCR and sequencing*

All cultures gave positive results for *P. perurans* when tested by *P. perurans* specific qPCR (Fringuelli *et al*., 2012). All cultures yielded products of expected size of approximately 630 nucleotides when DNA amplified by *P. perurans* specific primers (Young *et al*., 2008) in traditional PCR reactions. Sequencing of the 18S rDNA regions confirmed *P. perurans* when sequences were compared to data on GenBank. A number of ambiguous bases indicating nucleotide heterogeneity within the ribosomal DNA were observed.

*Generation of amoebic gill disease by clonal cultures and differences in virulence*

Five fish screened prior to challenge for the presence of a range of viral and bacterial pathogens, and for *P. perurans* and signs of AGD, were found to be negative.

Gills from the five control fish, exposed to filtered amoebae culture media, showed absence of gross lesions indicative of AGD or other insults.  Histological analysis of gill sections from the control fish confirmed absence of amoebae and pathological signs associated with AGD.  Some minor sloughing was observed and considered to be with normal range for this species.

All 5 clonal cultures tested, as well as the polyclonal culture, caused development of AGD in salmon during the experimental challenge. Differences in gross gill-arch score between the clonal cultures are categorised as statistically significant. The least effective clones at inducing AGD in Atlantic salmon at doses of 500 L-1 and 5000 L-1 were clones B9 and CE6 respectively (Fig. 3b, Table 1). B8, at both the 500 L-1 and 5000 L-1 dose, was the most effective clonal culture at generating AGD lesions and this was the only culture generating a gross gill-arch score of 5 (severe gill damage) at any dose.

Gill scores for different cultures based on histological assessment of the first gill arch gave similar results (data not shown). In summary, there are differences in gill damage between cultures and between challenge doses, with higher gill scores/greater gill damage at higher doses. However, the same trend with respect to the cultures generating most and least damage was seen for both doses.

*Distribution of lesions between different gill arches.*

A plot of the gross gill arch scores (indicative of gill damage) for each gill arch for both *P. perurans* infection doses is presented below (Fig. 4). Differences in gross score between gill arches are not categorised as statistically significant. A posteriori analysis indicates that the analysis would have been able to detect a consistent increase in gross score of 0.5 for one of the four gill-arches with a power >95%. In summary, there is no evidence of consistent variation in gross score greater than 0.5 between gill-arches.

*Comparison of disease scores based on histopathology and on gross clinical signs*

A display of the concordance between gross and histology scores from the first gill-arch from different sides of individual fish (Fig. 5a) and between mean gross gill score across all gill arches in right branchial chamber, and histology score from the first gill arch, left branchial chamber, of the same fish (Fig. 5b), are presented below). The value of G, 0.83, describing the association between gross and histology gill scores from first gill arch of right and left branchial chamber respectively, is categorised as being statistically significant from 0, indicating a positive association between histological and gross gill arch scores for assigning AGD severity to fish. This value is likely to be an underestimate of the true relationship because histological and gross scores were taken on the left and right gill arch respectively. A similar G value (0.84), also categorised as statistically significant, was observed for the association between mean gross score across all gill arches in right branchial chamber and histology score on the first gill arch, left branchial chamber.

*Comparison of amoebae load with gill score*

There is an apparent increase in the proportion of samples from which amoeba DNA was amplified with an increasing first gill-arch gross score (Table 2) and this association is supported by the results of a Generalised Linear Model. Inspection of the *P. perurans* qPCR Cp values revealed eight failures of amoeba DNA amplification from the first gill-arch (right branchial chamber) from the 60 challenged fish (13%), and amoeba qPCR Cp values of greater than 35, regarded as the upper Cp threshold for reliable detection, for a further 10 fish (17%).

The preponderance of Cp values greater than 35, and the association between the proportion of samples from which amoebae DNA was amplified with increasing gill score (Table 2), is consistent with the idea that non-detection is a consequence of a quantity of amoebae DNA below the detection threshold of the assay rather than a technical failure of the assay per se. A plot of the first gill-arch gross score and detected log10 *P. perurans* DNA quantity is displayed in Fig. 6. This plot, which excludes the 18 individuals for which amoeba DNA was not amplified, indicates an increase in gross gill-arch score with an increase in amoebae load. The value of G, 0.69, is categorised as being statistically significant from 0. A similar value of G of 0.57, also statistically significant from 0, characterises the association of mean gross gill arch score across all gill arches of right branchial chamber and amoebae load. In summary there is evidence of a positive association between gross gill arch score and amoeba DNA quantity and this is consistent with a relationship with *P. perurans* burden and disease severity.

**DISCUSSION**

Flow cytometry is used as a means to identify, characterise and sort different cell types and populations, and species within complex microbial communities, including isolation of single cells to generate clonal populations (Browne & Williams 1993; Hernlem & Ravva 2007; Guillebault *et al*., 2010; Boissière *et al*., 2012). Flow cytometry cell sorting is considered to be more reliable than limiting dilution approaches to obtaining single cells for clonal generation, and less laborious than micromanipulation (Ishii *et al*., 2010, Miao *et al*., 2010, Gross *et al*., 2015). Flow cytometry was used here to generate pure clonal cultures of *P. perurans* from single cells, isolated from an amoebae culture originating from infested salmon gills. A high degree of success was achieved with >50% of sorted cells giving rise to cultures. It is likely that even higher success rates could be obtained depending on the starting population of amoebae, and their physiological status and age. Analysis of autofluoresence indicated that *P. perurans* was autofluorescent under UV and violet excitation and was distinguishable from bacterial species within the analysed culture. This may prove useful in further studies of *P. perurans* using flow cytometry where autofluoresence as well as size can be used to separate *P. perurans* from bacterial populations, particularly in conditions where amoebae may be reduced in size due to biological/physiological state, or bacteria may form clumps due to sample processing. The use of seawater as a support medium for the amoebae did not affect the electrostatic charging of droplets containing individual amoebae, necessary for the sorting process. With respect to sorting of single *P. perurans* into a receptacle containing seawater for continued culturing, the sheath buffer used is not critical as the single drop of buffer will not affect the overall sea water composition in the receptacle. However, for other applications where high numbers of *P. perurans* are required to be sorted from contaminants into a single receiving container, then the sheath buffer should be replaced with sterile sea water or other media demonstrated to support viable *P. perurans* (data not shown).

All of the five clonal cultures established from flow cytometry-sorted single cells, and maintained for use in *P. perurans* studies, caused AGD in challenged salmon. Differences in virulence, as judged by resulting gill scores, were observed between some cultures at a dose of 5000 amoebae L-1 with culture CE6 showing lower, and B8 higher, virulence than the polyclonal and other clonal cultures. Similar results were observed at a dose of 500 amoebae L-1 except that clone B9 displayed a similar reduced virulence to that of CE6. As this study set out only to determine if clonal cultures isolated by flow cytometry could generate AGD in salmon, only one group of five fish per clone/amoebae concentration was used in challenges, with no replicates of treatments. It cannot conclusively be said that the differences observed per amoeba dose were due to inherent virulence differences in clones rather than to tank effect or culture preparation effects. However, exposure at both doses, showed the same virulence patterns for the clones, i.e. B8 and CE6 were classified as generating most and least severe lesions respectively for both doses. In a subsequent challenge experiment (not presented), both B8 and CE6 showed decreased virulence, as observed previously in long term maintenance of amoebae in culture (ref). Though reduced in magnitude, the clone B8 still showed more severe histopathology compared to CE6 (Ottavia Benedicenti, personal communication). Crosbie *et al*. (2010) found differences between different batches of *P. perurans* isolated from aquarium maintained infested donor fish in their ability to generate AGD gill scores. However, they suggested that the differences were likely to have originated from differences in the health of the amoebae population or their state of cell division at time of isolation. *Paramoeba perurans* is considered a facultative pathogen, being able to survive in the environment away from fish hosts. It is possible that *P. perurans* isolated from AGD-affected fish gills has already been selected for in terms of virulence, as seen in other pathogen models (Faruque *et al*., 2004). However, within pathogenic strains, clonal variation can still occur (Garin *et al*., 2001). It should also be noted that the differences in virulence, if real, may not be stable, or at least at the expression level, if not genomic. Bridle *et al*. (2015) demonstrated decreasing virulence in a *P. perurans* clone over time with loss of virulence after 1095 days in culture. Loss of virulence in pathogens, including amoebae, passaged *in vitro* is common (Dorson et al., 1978; Jellet & Scheibling, 1988; Ram *et al*., 1992; Song *et al*., 2005; De Marco Veríssimo *et al*. 2013; Songe *et al*. 2014). Virulence in the context of AGD may be effected in numerous ways, including higher proliferation of amoebae on gills, longer duration, better attachment or inability of host response to clear amoebae, production of virulence factors on the amoebae surface or secreted which interact with host. The avirulent clonal *P. perurans* culture presented in Bridle *et al*. (2015) demonstrated loss of attachment *in vitro*, and filtered media from the cultures lacked ability to induce lysis in monolayers of the Chinook salmon embryo cell line, CHSE-214, when compared to wild type *P. perurans,* recently isolated from fish. Nielsen *et al*. (2016) reported the presence of an amorphous matrix at the *P. perurans*-host membrane interface which they suggested indicated a role for extracellular products in generating pathology. Comparison of average gill score and average amoebae values, represented by *P. perurans* qPCR detection values normalised for *Salmo salar* ELF, representing the amount of mucous sampled, showed a significantly positive relationship between gill score and number of amoebae detected on the gills. Therefore, the clonal culture generating the highest average gill score (B8) was represented by higher numbers of amoebae on the gills. This may indicate that the virulence of this clone is linked to factors influencing amoebae numbers, such as growth rate, attachment ability, or resistance to an eliminating host response, rather than virulence factors differentially expressed by the individual amoebae of different clonal cultures acting directly with host to generate more severe gill lesions. Further studies are required to confirm the differences in virulence observed between the *P. perurans* clones.

Higher gill scores were observed with doses of 5000 amoebae L-1 compared to 500 amoebae L-1. This is in agreement with other studies where increasing amoebae numbers resulted in increased severity and numbers of gill lesions (Zilberg *et al*., 2001; Morrison *et al*. 2004). No pattern was observed with respect to gross gill score and specific gill arch. Differences in manifestation of the disease/attachment of amoebae between gill arches is an important consideration in relation to sampling for diagnostic purposes or as part of an epidemiological study. However, with respect to both situations, results for salmon and/or in an aquarium challenge, may not be representative for farmed/wild salmon nor other fish species, given differences in environment, behaviour, and in gill chamber structure.

Analysis of qPCR results, for detection of *P. perurans* in fish exposed to *P. perurans*, indicated that, at scores of 0-1, the qPCR assay as performed here i.e. single gill swab, DNA extraction and amplification of 18S rDNA, may be insufficiently sensitive to reliably detect amoebae presence. However, the ethanol, in which the swabs were stored, was not spun down and amoebae may have been lost, resulting in poorer detection, unrelated to the QPCR assay itself. As more information becomes available on the sensitivity of detection of different methods, this can be used to modify approaches, or qualify results, in monitoring and epidemiological studies.

A strong positive association was found between gill score based on histopathology of a single gill arch and the gill score, based on gross pathology, of the same single gill arch, and the average gross gill score of the four gill arches (an average gill score is often used on farms), from the opposite branchial chamber. This reflects previous findings (Adams *et al*., 2004) where agreement based on Kappa values between histology on a single arch versus gross clinical signs on any arch ranged from 0.52 – to 0.74. Disagreement between gross clinical and histopathological findings was considered by Adams *et al*. (2004) to be due to a number of factors including variation in sampling and preparation, reader error, gross signs not being attributable to *P. perurans*, and, early stage lesions, detected by histology, not having gross manifestation. Despite the requirement to kill fish, and the effort required, histological examination was recommended to obtain a more robust diagnosis for AGD. It was also recommended where possible to choose for analysis a gill arch with lesions rather than a fixed agreed arch. As found in studies by Adams *et al*., (2004), Clark & Nowak 1999 and Zilberg *et al*., 2001, our results also indicate that agreement between gross and histological analyses is lower for less severe gill scores. We found that for many scores of 1-2 obtained based on histopathology, gross gill scores of “0” were obtained. Therefore, for early detection of AGD, as with the qPCR DNA based approach described above, gross pathological examination is not always reliable.

In summary, flow cytometry is a useful method to isolate clonal cultures of *P. perurans*, the clonal cultures can cause AGD in challenged salmon, and putative differences in virulence were observed between the clones isolated. This latter finding promises the potential of material to study virulence factors in *P. perurans*. Flow cytometry and cell sorting may be particularly useful if variation in parameters at an individual amoeba level within a population is of interest, as it is substantially faster than micromanipulation approaches and considered more reliable than the dilution method.

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**Tables**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Dose | Cultures | Mean gill scorea  median (range) | | Amoeba loadb  median (range) | |
| 500 | B8 | 1.8 | (1.0,2.2) | 0.038 | (undetc-0.165) |
| Polyclonal, C6, CC3 | 0.8 | (0.2,1.5) | undet | (undet-0.028) |
| B9, CE6 | 0.5 | (0.0,1.0) | undet | (undet-0.007) |
| 5000 | B8 | 3.8 | (3.5,4.5) | 0.136 | (0.019-0.482) |
| Polyclonal | 2.8 | (2.5,3.5) | 0.023 | (0.012-0.113) |
| C6,CC3,B9 | 2.0 | (0.8,3.0) | 0.007 | (undet-0.133) |
| CE6 | 1.0 | (0.5,1.7) | 0.002 | (<0.001d-0.017) |

Table 1. Summary of gross gill arch scores and amoebae loads for different clonal cultures/groups of cultures. aThe median of mean gill-arch scores for individuals within groups. b*P. perurans* load is represented by qPCR Cp values converted to estimated target amounts (ETAs) and normalised to host ELFα ETAs; camplification with a Cp of >35; drelative amplification with a Cp of <35 but less than normalised *P. perurans* ETA of 0.001

|  |  |  |
| --- | --- | --- |
| **Gross score** | **Number of individuals from which *P. perurans* DNA** | |
| **amplified** | **not amplified** |
| 0 | 2 | 6 |
| 1 | 12 | 10 |
| 2 | 14 | 2 |
| 3 | 9 | 0 |
| 4 | 5 | 0 |
| 5 | 0 | 0 |

Table 2. Number of individuals from which *P. perurans* DNA was amplified at each gross score for first gill arch of right branchial chamber.

**Figure legends**

Figure 1. Scatter (A) and UV parameters (B) on BD Influx™ were used to differentiate and isolate different populations (P1 – P5) within the *Paramoeba perurans* culture. P1 and P2 identified microscopically as amoebae, the latter representing smaller and rounded amoebae.

Figure 2. Clonal culture (x 20) in 96-well plate approximately one week following sorting of single amoeba cells using flow cytometry and cell sorter.

Figure 3. Mean gross gill-arch score of individual fish for each culture at challenge doses of a) 500 and b) 5000 amoebae L-1. The mean score was obtained by averaging the scores for each of the four gill arches of an individual fish. Cultures not linked by a horizontal line (below axis labels) differed in their ability to generate gill lesions. A horizontal jitter around cultures has been incorporated to improve the visualisation of values.

Figure 4. Severity in gill damage across gill arches at challenge doses of a) 500 *P. perurans* L-1, b) 5000 *P. perurans* L-1.

Figure 5. Comparison of gross and histology gill scores with histological scoring from the first arch from the left branchial chamber and a) the gross gill score represented by the first gill arch from the right branchial chamber and b) the mean gross gill score of all four gill arches from the right branchial chamber. A horizontal jitter has been incorporated around histology scores of 1, 2, 3, 4 and 5.

Figure 6. Comparison of gross gill score, from first gill arch of right branchial chamber, and detected log10 *P.* *perurans* DNA.