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Succinctus

Neoparamoeba perurans loses virulence during clonal culture

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ABSTRACT

Amoebic Gill Disease affects farmed salmonids and is caused by *Neoparamoeba perurans*. Clonal cultures of this amoeba have been used for challenge experiments, however the effect of long-term culture on virulence has not been investigated. Here we show, using in vitro and in vivo methods, that a clone of *N. perurans* which was virulent 70 days after clonal culture lost virulence after 3 years in clonal culture. We propose that this is related either to the lack of attachment to the gills or the absence of an extracellular product, as shown by the lack of cytopathic effect on Chinook salmon embryo cells. The avirulent clonal culture of *N. perurans* allowed us to propose two potential virulence mechanisms/factors involved in Amoebic Gill Disease and is an invaluable tool for host-pathogen studies of Amoebic Gill Disease.

Amoebic Gill Disease (AGD) affects salmonids farmed in the marine environment worldwide, with the exception of Canada, and its significance has increased over the last few years (Nowak, 2012). This disease has been reported from other farmed fish species including turbot, *Scophthalmus maximus*, (Mouton et al., 2014) and ayu, *Plecoglossus altivelis*, (Crosbie et al., 2010). The causative agent of AGD is *Neoparamoeba perurans* as shown by molecular evidence (Young et al., 2007) and fulfillment of Koch's postulates (Crosbie et al., 2012). AGD is initiated by the association, and presumably attachment, of *N. perurans* to the gill epithelia (Lovy et al., 2007) which results in extensive gill epithelial hyperplasia and proliferation of amoebae associated with the hyperplastic lesions (Adams and Nowak, 2001). As *N. perurans* is a free-living amoeba and an opportunistic pathogen, AGD can be transmitted horizontally when *N. perurans*-containing mucus is sloughed off infected gills into the water column and the amoebae are free to colonise the gills of fish (Nowak et al., 2014). Despite approximately 30 years of AGD research, relatively little is known regarding how these free-living amoebae interact with the gills of naïve fish and induce AGD. Research concerning the host-pathogen-environment interaction involved in AGD has until recently been biased towards the host and potential immunity to AGD (Nowak et al., 2014). For many years the situation was further complicated by ambiguity surrounding the causative agent of AGD until research by Young et al. (2007) definitively identified and named the agent as *N. perurans*. Furthermore, it was not until an in vitro culture method was established by Crosbie et al. (2012) that Koch's postulates could finally be fulfilled and a concerted effort made towards investigating the role of *N. perurans* and its association with the host during AGD. While it is now possible to culture *N. perurans* in vitro (Crosbie et al., 2012) co-habitation still underpins the majority of experimentally induced *N. perurans* infections and AGD studies (Nowak, 2012) and little progress has been made towards identifying virulence factors and mechanisms by which *N. perurans* causes AGD. To fulfil Koch's postulates we previously showed that *N. perurans* clone 4 was virulent 70 days after establishing the clonal culture (Crosbie et al., 2012). This clone has been in a continuous culture since February 2011 and in a clonal culture since May 2011. Importantly, further investigation suggested that clone 4 was less virulent after 27 months in clonal culture (Crosbie et al., 2014). Here, we show that clonal *N. perurans* loses virulence in culture over time, providing an invaluable tool to identify and characterise potential virulence factors of *N. perurans*.

To investigate virulence in an in vitro system we used the Chinook salmon embryo cell line. CHSE-214 cells were cultured in L-15 growth medium supplemented with Glutamax (Invitrogen, Victoria, Australia) and 10% foetal bovine serum (Invitrogen) held at 18 °C under normal atmospheric conditions.

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For subsequent experiments, confluent monolayers were lifted using 0.25% Trypsin/EDTA (Invitrogen) and cell concentration was adjusted to approximately 1.2×10^5 cells/mL with L-15 growth medium. Aliquots of 1 mL were used to seed each well of 12-well tissue culture plates (Iwaki, Tokyo, Japan) yielding monolayers of approximately 1×10^5 cells/well. For image analysis, glass coverslips were placed in each well of a 6-well tissue culture plate (Iwaki) prior to the addition of 2 mL of cell suspension. All cultures were incubated in L-15 growth medium for 48 h prior to experimental manipulations.

Wild type *N. perurans* were freshly isolated from the gills of cultured Atlantic salmon, *Salmo salar*, held at the University of Tasmania Aquaculture Centre (Launceston, Tas, Australia) and clonal (clone 4) *N. perurans* were cultured on malt yeast agar (MYA) plates as previously described (Crosbie et al., 2012). Clonal *N. perurans* (clone 4) trophozoites had been in culture on MYA plates at the University of Tasmania for more than 3 years and 200 passages (Crosbie et al., 2012). To assess the effect of salinity on CHSE-214 survival and amoeba attachment, L-15 growth medium was adjusted to 20, 25 and 30 practical salinity units (psu) by the addition of 0.2 μm filtered sea water (35 psu) and used to culture CHSE-214 cells and *N. perurans* trophozoites in quadruplicate wells for 24 h at 18 °C. After cell and amoeba counts were obtained at 24 h, 1 mL of the culture medium was removed from wells containing 0, 1×10^2, 1×10^3 and 1×10^4 amoebae/well of wild type or clonal amoebae cultured in L-15 growth medium at 20 psu. The culture media were centrifuged at 400*g* for 5 min at 4 °C, then filtered through a 0.2 μm polyethersulfone filter (Membrane Solutions, Texas, USA) to obtain cell-free supernatants that were then added as 1 mL volumes to CHSE-214 cells and incubated for 24 h at 18 °C. CHSE-214 cell survival after 24 h culture in L-15 at 20 psu with supernatants from wild type and clonal amoebae was assessed using a one-way analysis of variance (ANOVA) using Tukey's Multiple Comparison test with a significance level of $P < 0.05$. Amoeba attachment at different salinities was statistically assessed using a two-way ANOVA using Bonferroni post tests with a significance level of $P < 0.05$.

To assess *N. perurans* virulence in vivo we followed methods used previously (Crosbie et al., 2012) with the following modifications. Briefly, triplicate tanks filled with sea water (salinity 35 psu) were used for each infection study using *N. perurans* clone 4 or the wild type *N. perurans* and one tank was used as a negative (uninfected) control. Twenty fish were stocked in each tank. Fish were challenged with *N. perurans* (3000 cells L^{-1}) after 5 days of acclimation and five fish from each tank were removed at 7, 14, 21 and 28 days post-challenge for analysis. In addition, a 1 L water sample was taken from each tank on each sampling day and analysed for the presence of *N. perurans* using quantitative real time PCR (qPCR) as described (Supplementary Method S1). Fish used in this trial were approved for experimentation by the University of Tasmania, Australia, Animal Ethics Committee (approval number A0013938). Statistical analyses using one-way ANOVA and two-way ANOVA, and graphing were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, California, USA).

In vitro experiments showed that, in contrast to wild type *N. perurans*, after 3 years in continuous culture and 200 passages the supernatant generated from *N. perurans* clone 4 culture had no cytopathic effect (CPE) on CHSE-214 cells (Figs. 1D and 2). Salinity experiments showed that, while CHSE-214 cells had no loss of viability in L-15 growth medium when cultured at 20 psu for 24 h (Fig. 1A), the percentage of attached amoebae at this reduced salinity level was significantly less compared with the percentage of attached amoebae when cultured at 30 psu (Fig. 1C). Loss of amoeba attachment at reduced salinity was not detrimental or indicative of amoeba viability and was shown to be completely reversible once the non-adherent amoebae were returned to sea water at 35 psu (data not shown). Similar to the in vitro findings, the *N. perurans* clone 4 was not virulent in vivo as determined by histology of gills from fish exposed to *N. perurans* clone 4. No AGD lesions or amoebae were observed in the gill sections of fish exposed to clone 4, indicating that clone 4 was not virulent after 3 years in clonal culture despite being virulent after 70 days in clonal culture (Crosbie et al., 2012). The loss of virulence in *N. perurans* clone 4 appears to be dependent on the time in culture as clone 4 had reduced virulence after 639 days in clonal culture; virulence was further reduced at 869 days and a total loss of virulence was observed at 1095 days or 3 years in clonal culture (Table 1). Furthermore, the absence of disease in fish challenged with *N. perurans* clone 4 was not due to an insufficient challenge dose. In tanks inoculated with 3000 wild type or clone 4 *N. perurans*/L, both contained less than 10 amoebae/L on day 7 post-challenge and throughout the remaining 3 weeks of the in vivo challenge parasite numbers fluctuated between 0 and 28 amoebae/L. Finally, at the end of the 4 week challenge period seven amoebae/L were detected in the tanks inoculated with *N. perurans* wild type and four amoebae/L in tanks inoculated with *N. perurans* clone 4. These results indicate that while the numbers of viable *N. perurans* decreased soon after the beginning of challenge, there were still sufficient numbers of both *N. perurans* wild type and clone 4 over the challenge period and they were at comparable concentrations. Furthermore, the concentrations of both *N. perurans* wild type and clone 4 in the challenge systems were equivalent to those reported in AGD-affected commercial salmon cages in Tasmania (Wright et al., unpublished data).

Other pathogenic marine amoebae have been reported to lose virulence during culture. For example, in comparison to mortality rates of controls exposed to *Paramoeba invadens* maintained in vivo, exposure of sea urchin, *Strongylocentrotus droebachiensis*, to a 15 week old monoxenic culture of *P. invadens* did not induce any mortality, while challenge with a 58 week old polyxenic culture of *P. invadens* delayed mortality of sea urchins by 5 days (Jellett and Scheibling, 1988). Loss of virulence in culture is well documented for isolates of several free-living species of amoebae pathogenic to mammals (Kasprzak et al., 1974). Furthermore, virulence of an *Acanthamoeba* strain could be restored when the amoeba was passaged through mice (Mazur and Hadaś, 1994). *Naegleria fowleri*, another well studied free-living amphizoic amoeba that causes primary amoebic meningoencephalitis in humans, was also attenuated when maintained in prolonged axenic culture but virulence was restored following serial passage in mice (Marciano-Cabral and Cabral, 2007).

Reduced CHSE-214 cell survival following addition of culture supernatant from wild type compared with survival in supernatant obtained from the avirulent clone 4 clearly implicate an extracellular product (ECP) is responsible for the CPE associated with the wild type *N. perurans* (Figs. 1 and 2). Based on our findings we propose the ECP produced by *N. perurans* is a virulence factor associated with AGD. An ECP from *Neoparamoeba* sp. (isolated from AGD affected gills) also showed a CPE in a salmon cell line (Butler and Nowak, 2004). The production of ECPs by free-living amoebae that induce host-cell cytopathic effects in vitro is well documented (Serrano-Luna et al., 2013). Free-living amoebae such as *Acanthamoeba* spp. and *N. fowleri* are known to produce proteolytic ECPs that contribute to the pathogenesis associated with these two opportunistic pathogens (Aldape et al., 1994; Mitro et al., 1994). Extracellular proteases may directly destroy tissues or degrade protective barriers composed of mucins or proteins released during the host immune response (Serrano-Luna et al., 2013). However, the relationship between ECPs and virulence is often complex and may not be as straightforward as the presence or absence of a particular ECP. In comparison to the human

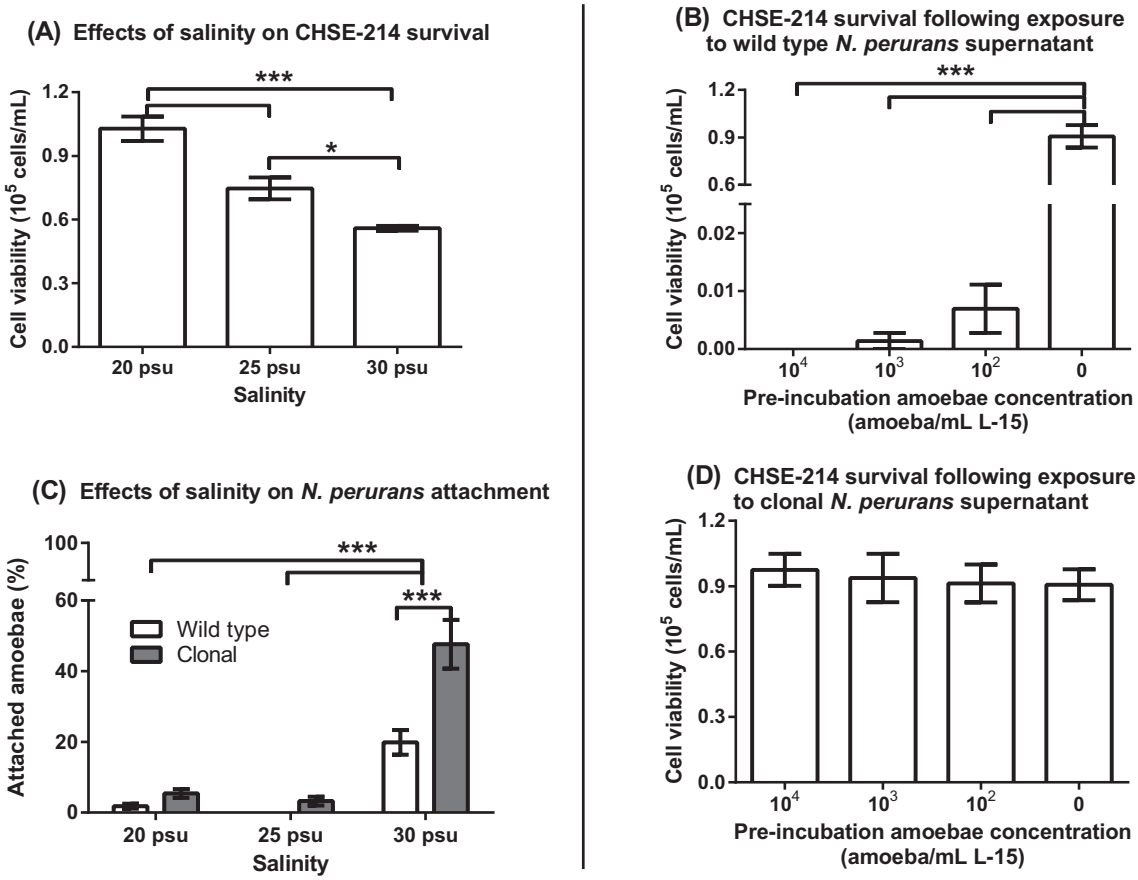

Fig. 1. In vitro experiments showing percentage attachment of *Neoparamoeba perurans* and the effect of *N. perurans* culture supernatants on Chinook salmon embryo cell line. (A) Percentage *N. perurans* attached at different salinities in L-15 culture medium. (B) Chinook salmon embryo cell line cell survival after culture in L-15 at 20 psu for 24 h with culture supernatants from wild type *N. perurans*. (C) Effect of salinity on *N. perurans* attachment. (D) Chinook salmon embryo cell line cell survival after incubation in L-15 at 20 psu for 24 h with culture supernatants from clonal *N. perurans* (clone 4) Significance of Chinook salmon embryo cell survival (B, D) was assessed by a one-way ANOVA and amoeba attachment under varying salinities (A, C) using a two-way ANOVA ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

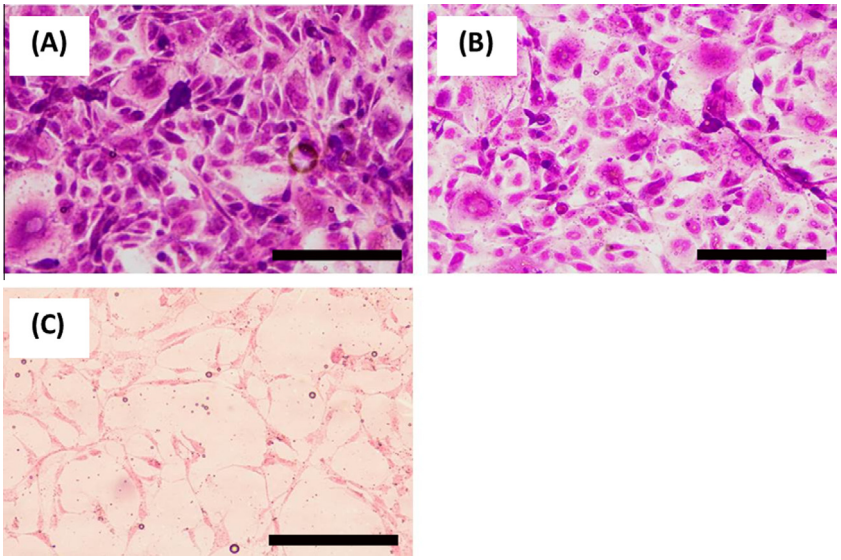

Fig. 2. Effects on salmon embryo cell line following 24 h incubation in: L-15 medium alone (A), L-15 medium previously incubated for 24 h with *Neoparamoeba perurans* clone 4 (B), L-15 medium previously incubated for 24 h with wild type *N. perurans* (C). A cytopathic effect on Chinook salmon embryo cell line cells is visible following exposure to wild type *N. perurans* (C). Scale bar = 50 μm.

pathogen *Entamoeba histolytica*, the morphologically identical but non-pathogenic *Entamoeba dispar* expresses the same ECPs but at a much reduced concentration (Bruchhaus et al., 1996). Furthermore, loss of virulence in an *Acanthamoeba* sp. correlated

Table 1
Comparison of the virulence of *Neoparamoeba perurans* after different duration of clonal culture. The duration of exposure is shown as well as the percentage of fish (Atlantic salmon) with Amoebic Gill Disease lesions including presence of paramoebae, detected in histological sections. In all studies, fish were exposed to approximately 3000 *N. perurans* amoebae/L (only clone 4 data are shown in the table). Amoebic Gill Disease lesions with amoebae were present in all positive controls (fish exposed to wild type *N. perurans*) and absent from all negative controls (unexposed fish). Salinity of the water in all tanks was maintained as 35 psu, and the temperature at 16–17 °C. Water exchanges were performed as required to maintain optimum water quality.

Time in clonal culture (days)	Duration of exposure (days)	Percentage of fish with AGD lesions (based on histology) (%)	References
70	37	60	Crosbie et al. (2012)
639	35	33	Crosbie et al. (2014)
869	48	8	Crosbie et al. (2014)
1095	28	0	This paper

with a reduced proteolytic profile of the attenuated amoebae (Verissimo et al., 2013).

The in vitro CPE identified here and its relevance to AGD is unclear given that extensive histological studies on AGD-affected salmon revealed no evidence of cytolytic activity (Adams and Nowak, 2001). While potential damage to host tissues may be masked by the exuberant proliferative host response it seems likely that the CPE demonstrated in vitro is evidence of an ECP with a less direct virulence mechanism. Alternatively, the CPE observed in vitro using the CHSE-214 cell line may be unrelated to AGD pathology. Importantly, amoeba attachment is likely to impact AGD development as we found that although both wild type and clonal *N. perurans* were present in the water column throughout the challenge period; unlike the wild type amoebae, no amoebae were found attached to the gills of salmon challenged with clone 4. Whether or not the lack of attachment by clone 4 is related to the absence of the ECP as shown in vitro is unclear. Further research investigating this relationship is warranted given the established proteolytic activity of several amoebic ECPs that promote attachment to the host (Serrano-Luna et al., 2013). Nonetheless, whether dependent on an ECP or some other attachment factor, the in vivo challenge results clearly imply amoeba attachment as necessary for AGD.

To the best of our knowledge this is the first report showing that *N. perurans* can lose virulence in clonal culture. While the *N. perurans* clone 4 lost virulence after 3 years, we do not know how variable *N. perurans* isolates are with respect to loss of virulence in culture nor do we know if virulence can be restored. In conclusion, we have established an avirulent clone of *N. perurans* that has allowed us to identify two potential virulence mechanisms involved in AGD. It is envisaged that future studies using this avirulent clone will identify additional virulence factors and allow further characterisation of the host-pathogen interaction in AGD.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2015.04.005.

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